STUDIES OF THE RAW EGG WHITE SYNDROME IN RATS [1]

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ONE FIGURE

(Received for publication November 9, 1933)

A novel syndrome occurring in rats which received Chinese dried egg white as the dietary source of protein was reported by Boas ('27). The chief symptoms were a characteristic 'eczematous dermatitis,' accompanied by alopecia, blepharitis, spasticity, and in some cases by edema of the feet. Skin hemorrhages were seen in severe cases. The similarity of the symptoms to those of pellagra was recognized. The condition was prevented by inclusion of one of the following substances in the diet: raw potato starch, arrowroot starch, dried yeast, fresh egg white, egg yolk, milk, commercial casein, crude lactalbumen, spinach, cabbage, banana, or dried horse serum. The cereal starches, wheat bran, marmite, malt extract, butter, or raw meat failed to prevent the occurrence of the symptoms. The syndrome did not develop, however, if the egg white was cooked before drying. In a later paper (Fixsen, '31) the same author[2] presented data to support the hypothesis that a toxic substance formed during the drying process caused the disease. It was said that ovalbumen, ovoglobulin, or the total albumen and ovomucoid fraction prepared from crude egg white furnished satisfactory protein for young rats and that the nutritive properties of these fractions were not altered by desiccation as was the case with the crude egg

[1] Published with the permission of the director of the Alabama Experiment Station.

[2] Mrs. Boas became Mrs. Fixsen.

white. Moreover, it was possible to prepare, from dried egg white, fractions which were non-toxic, by precipitation with ammonium sulfate.

The earlier results of Boas were confirmed by Findlay and Stern ('29) who reported that raw egg white was protective and that raw liver had a high protective potency. These investigators suggested that the egg white syndrome in rats is the analogue of pink disease (Swift's disease, erythredema) in children.

Parsons ('31) has studied the effects of diets containing 20 to 66 per cent of egg white. She has observed, in addition to the symptoms observed by Boas, a high rate of mortality in young rats soon after they were placed on the egg white diets. This was largely prevented by the feeding of liver either before or after the rats were weaned.

In three papers which appeared after the completion of the experiments reported in the following pages, Parsons and co-workers have discussed additional findings. They (Parsons and Kelly, '33) have concluded: A) that the tendency to produce a pellagra-like condition in rats is a property of native egg white rather than a result of desiccation; B) that the injury involves an interrelation between a positive toxicity and a relative absence of a protective factor; and, C) that the toxicity of the native egg white is destroyed by heat. They also found ('33) that the injurious property of the egg white was destroyed by incubation with acid pepsin or mild hydrochloric acid alone but persisted in egg white denatured with alcohol. The protein precipitated by complete saturation of egg white solution with ammonium sulfate was found to be toxic. In addition, it was observed (Parsons, Lease and Kelly, '33) that dried yeast, dried egg yolk, wheat embryo and dried milk were only moderately protective, a concentration of one to three times that of the 'toxic' egg white in the diet being necessary for protection. Cooked beef liver, pork liver, and beef kidney equivalent to one-fourth of the egg white in the diet were curative. Spleen, heart, ovaries, adrenals, blood, hemoglobin, or liver extract no. 343 (Lilly) had little or no

potency. The activity of liver, which was said not to depend on its nucleo-protein fraction, was decreased by boiling for 1 hour with 5 per cent HCl and destroyed by heating cooked liver 6 days at 100°.

Ringrose, Norris and Heuser ('31) have reported "lesions of a distinctly pellagrous character" in chicks which received diets containing dried egg albumen.

On the other hand, Chick, Copping and Roscoe ('30) have found that 2.5 to 5.0 gm. of fresh, cooked egg white (dry weight 0.3 to 0.6 gm.) cured the skin lesions and restored 'normal' growth in rats receiving a diet deficient in vitamin G (B_2). The rate of growth was not maintained, however, when egg white was the sole source of vitamin G.

The similarity of the symptoms of the egg white disease to the symptoms produced by Salmon ('31) in rats receiving diets in which the casein and the yeast had been subjected to dry heat treatment led to a study of the egg white syndrome in this laboratory. The results of the study are reported in the following pages.

EXPERIMENTAL METHODS

Animals and their care. A mixed strain of albino-hooded rats was used. In preliminary experiments with egg white diets it was found that, when young rats were transferred to the experimental diets at weaning time and given food ad libitum, a high rate of mortality resulted. This was caused by an acute hemorrhagic nephritis which usually developed in the period from the tenth to the fifteenth day of the experiment. It was apparently the condition which has been referred to by Parsons as the 'initial injury' resulting from egg white diets.

For the experiments reported in this paper a special routine was developed which largely avoided this early injury. After the young were 17 days old, the dam was removed from the maternity cage each morning about 7 to 8 o'clock and placed in a separate feeding cage where she had access to the stock diet. At the same time the experimental diet was placed in

the cage with the young. The dam was returned to the maternity cage about 10 to 11 o'clock, left with the young for approximately 3 hours, and was again removed to the feeding cage until late afternoon when she was returned to the maternity cage for the night. The experimental diet was always removed when the dam was with the young. The young were weaned at 27 days of age and placed on experiment. They were started on 3 gm. of the experimental diet per rat daily. The diet was slowly increased until at the end of 2 weeks it was being supplied ad libitum. Fresh portions of the diets were placed in the feed jars and distilled water was supplied in clean vessels each day. Individual cages with raised screen bottoms were used in all of the experiments.

Experimental diets. The composition of the experimental diets is shown in table 1. The raw, dried egg white was a Chinese product in the form of large yellow flakes, 'crystals.' It was completely soluble.

The cooked, dried egg-white was prepared by adding 3 liters of H_2O to 2 kilos of the dried egg white and leaving the mixture in the refrigerator until the material was completely dissolved. The solution was then heated in an electric oven for 30 minutes at 90 to 100°C. and the coagulated mass was spread in a thin layer and dried at 37°C.

The raw, fresh egg white was from fresh eggs obtained from the station poultry farm. The eggs were carefully broken and the whites separated from the yolks each day just before feeding.

The extracted egg white was prepared by pouring 11 liters of fresh egg white into 20 liters of boiling H_2O containing 50 ml. of concentrated HCl. The mixture was again brought to the boiling point, the coagulum was removed and, after the addition of 50 ml. concentrated HCl, another 11-liter batch of egg white was poured into the boiling extract. The extract was then drawn off. The two batches of coagulated albumen were combined and extracted by percolating with 51 per cent alcohol[3] until the extract was nearly colorless. A total of

[3] The strength of alcohol is expressed in this paper as per cent by weight.

TABLE 1

Composition of diets

Diet	A	A-1	B	B-1	B-2	C	C-1	C-2	C-3	C-4	D	E	F	G	H	I	J
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Raw dried egg white	18.0	18.0	66.0	66.0	66.0	18.0	18.0	18.0	18.0	18.0	18.0	12.0					
Raw fresh egg white															150.0[3]		
Cooked dried egg white														18.0			
Extracted egg white																18.0	
Extracted casein									10.0	18.0			12.0				18.0
Yeast residue		10.0		10.0	20.0		10.0										
Hydrolyzed yeast residue								10.0									
Ground white corn												85.0	85.0				
Sucrose	77.0	67.0	29.0	19.0	9.0	56.5	46.5	46.5	46.5	38.5	56.5			56.5	56.5	56.5	56.5
Salt mixture 186[1]	4.0	4.0	4.0	4.0	4.0	4.5	4.5	4.5	4.5	4.5	4.5			4.5	4.5	4.5	4.5
Agar	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0			1.0	1.0	1.0	1.0
Filtered butterfat						18.0	18.0	18.0	18.0	18.0				18.0	18.0	18.0	18.0
Cod liver oil[2]						2.0	2.0	2.0	2.0	2.0	2.0			2.0	2.0	2.0	2.0
Hydrogenated cottonseed oil											18.0						
Calcium carbonate												2.0	2.0				
Sodium chloride												1.0	1.0				

[1] J. Biol. Chem., 1927, lxxiii, 487.

[2] When cod liver oil was not incorporated in the basal diet, each rat received 0.10 ml. per day.

[3] The fresh egg white was added to each rat's weighed portion of the mixture of other constituents daily.

133 liters of extract, including the first aqueous liquor, was obtained. The coagulum was pressed as dry as possible in a hand press and placed in current of air from fan at 37°C. until nearly dry. The drying was completed in a forced-draft oven at 70°C. and the dried product was then ground in a burr mill. A total of 2060 gm. of the dry extracted material was obtained from the 22 liters (22.83 kilos) of fresh egg white.

The egg white extract was prepared by concentrating[3] the 133 liters of extract obtained from the coagulated egg white. When the volume was reduced to 12 liters the concentrated extract was cooled, shaken with ether, and the aqueous layer filtered through a fluted filter. The filtrate was then concentrated until 1 ml. was equivalent to 2 gm. of egg white (dry basis).

The brewer's yeast was Vita-Food No. 1, Red Label, pasteurized brewer's yeast. The baker's yeast was Northwestern dehydrated yeast.

The yeast residue was prepared from Vita-Food No. 1, Red Label, unpasteurized brewer's yeast. Forty-five kilos of this yeast were placed in an enamel-lined kettle and percolated with 51 per cent alcohol until a total of 500 liters of extract was obtained. Fifteen liters of 93 per cent alcohol were then poured over the extracted yeast to facilitate the dehydration and the material was sucked as dry as possible by vacuum. The extracted residue was then spread in a thin layer and dried in a current of air at 35 to 40°C.

The hydrolyzed yeast residue was the product resulting from adding 2.5 liters of a solution containing 100 ml. of concentrated HCl per liter to 500 gm. of the extracted residue described above and boiling on an asbestos bath for 5 hours. The excess HCl was neutralized with NaOH leaving the preparation just slightly acid to litmus. The preparation was then dried in a forced-draft oven at 70°C.

Extract B-G was obtained by concentration[4] of the first 400 liters of extract from 45 kilos of brewer's yeast until 1 ml.

[4] The concentration of extracts was always done under diminished pressure.

of the extract was approximately equivalent to 2 gm. of the original yeast. The concentrated extract was then shaken with ether to remove fatty material and after standing in refrigerator 2 to 3 weeks was filtered while cold through fluted filters. The volume was adjusted to make 1 ml. equivalent to 2 gm. of yeast.

Extract B was prepared by treating a yeast extract similar to the above with 1 gm. of English fuller's earth for each 23 to 30 gm. of yeast,[5] the extract having previously been adjusted to about pH 3.50. The adsorbate was filtered out, thoroughly washed with H_2O, dried, and the vitamin B extracted from the dried solid with pyridine-acetate in 80 per cent alcohol (Salmon, '31). The extract was concentrated and subjected to fractionation by increasing strengths of alcohol until a fraction soluble in absolute alcohol was obtained. The vitamin was then precipitated from aqueous solution by tannic acid, the precipitate dissolved in acetic acid, and the tannic acid removed with Pb acetate. The filtrate was concentrated to near dryness, the residue extracted with H_2O, and the solution deleaded with H_2SO_4. The filtrate was made to volume with 25 per cent alcohol and its vitamin B potency determined by testing on pigeons. This extract furnished a potent source of vitamin B but was free from vitamin G.

The dried liver was prepared by drying ground beef liver in a forced-draft oven at 70°C.

The liver residue was prepared by extracting fresh, ground beef liver with 51 per cent alcohol, allowance being made for the moisture in the fresh material. Extraction was continued until 65 liters of extract were obtained. The extracted residue was dried at 70°C.

The liver extract was obtained by concentrating the above 65 liters of extract to 3 liters, shaking with ether to remove fatty material, and concentrating the aqueous layer to 850 ml. After standing in a refrigerator for 10 days, the extract was

[5] The amount of fuller's earth used depended upon the potency of the yeast as determined by protective tests with pigeons.

filtered and the volume adjusted so that 1 ml. corresponded to 1 gm. of the material in the extracted residue.

The casein was extracted by percolating with water acidulated to 0.20 per cent with acetic acid for 1 week and then with 20 liters of 93 per cent alcohol per 45-kilo batch of casein to facilitate drying. The extracted casein was then sucked as dry as possible by evacuation and dried in current of air at 35 to 40°C. It was ground to pass through a 40-mesh sieve before being mixed into the basal diet. Tests showed this material to be free from vitamins B and G.

Duration of experiments. The length of the experimental period was 14 weeks, except in some cases where the animals died, or where they were used for curative tests, or where the outcome was obvious in a shorter period.

RESULTS

Basic raw egg white diets. Table 2 shows the weights of rats which received the basic raw egg white diets. Group 1 received diet A containing 18 per cent of raw, dried egg white and group 3 received diet B containing 66 per cent of raw, dried egg white. Both groups received sufficient extract B to furnish vitamin B in excess of the normal requirement. (Preliminary experiments confirmed the findings of Chick and co-workers ('29, '30) that egg white is lacking in vitamin B.) The behavior of the two groups was very similar. A rigid limitation of the food intake during the first week of the test was necessary to control diarrhea; as a result there was some loss in weight. Despite the gradual increase of food to ad libitum feeding by the beginning of the third week, the rats were unable to make appreciable gains on either diet. When extract B-G was substituted for extract B, the rate of growth was increased on diet A (group 2).

Since diet A was a low fat diet and was probably deficient in the unsaturated fatty acids which have been shown (McAmis et al., '29; Burr et al., '29, '30, '32) to be essential for normal nutrition, the effect of adding 0.20 ml. of linseed oil per rat daily was determined. This addition increased

the rate of growth significantly when extract B was fed (group 4) but increased it even more when extract B-G was fed (group 5).

The rate of growth on the medium fat diets C and D (groups 6 and 9) was comparable to that on diet A supplemented with linseed oil. Again the substitution of extract B-G for extract B produced a favorable response on the growth rate (groups 7 and 10). Diet C was not improved by the further addition of linseed oil (group 8). Growth was slightly improved by the addition of linseed oil to diet D (group 11) but severe skin lesions developed and an attendant decline in weight occurred earlier in the group which received the linseed oil; this was possibly associated with a slightly

TABLE 2

Average weekly weights of rats. Basic raw dried egg white diets

Group	1	2	3	4[3]	5[3]	6	7	8[3]	9	10	11[3]
Diet	A	A	B	A	A	C	C	C	D	D	D
Extract	B[1]	B-G[2]	B[1]	B[1]	B-G[2]	B[1]	B-G[2]	B-G[2]	B[1]	B-G[2]	B-G[2]
Number of rats	4	4	4	2	4	5	8	4	4	4	4
Week	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Initial	48	48	50	53	47	47	53	49	51	52	52
1	42	41	44	67	43	50	55	59	59	66	64
2	49	50	53	82	52	58	72	77	62	74	84
3	54	67	59	91	71	68[4]	91	94	74	83	102
4	58	80	69	96	87	76	110	109	81	99	114
5	62	91[4]	74	106	101	83	125	123	85	112	128
6	57[4]	91	70	108	106	88	139	123	89	122	135
7	66	100	70	113	118	88	145	128	94	128	139
8	61[4]	105	66	106	129	107[4]	146	134	90	131	132
9	68[4]	109	65	99	139	110	143	128	94	132	122
10	79	110	58	99	145	109	144	125	95	133	118
11	70	110	60[5]	97	151	104	141		97	133	119
12	75	110	58		155	115	140		100	133	116
13	75	100	63		158	122	139[4]				
14	70[4]	100[5]			152	123					

[1] In all cases where extract B-G was not given, sufficient extract B was fed to insure an adequate and uniform amount of vitamin B for each rat.

[2] When extract B-G was given each rat received a daily allowance representing the extract from 0.50 gm. brewer's yeast.

[3] Each rat in group 4, 5, 8 and 11 received 0.20 ml. of raw linseed oil daily.

[4] Indicates death of a rat.

[5] Indicates death of two rats.

larger food consumption and higher growth rate in the early part of the experiment. It should be borne in mind that the effect of correcting a deficiency in these diets may be apparent only in the early part of the experiment. Regardless of the correction of minor faults in the diets, the animals eventually decline.

Symptomatology. The major features of the symptomatology observed were similar to those described by Boas and by Parsons. In preliminary experiments a high rate of mortality occurred in some groups within the first 10 to 15 days of the experiment. Rats which were changed suddenly to diets containing as little as 18 per cent of raw, dried egg white and given food ad libitum usually showed no abnormal symptoms for 8 to 10 days, with the exception of some diarrhea. Then individual rats would suddenly refuse a large part or all of the day's allowance of food. These animals appeared extremely nervous and excitable for a few hours but gradually became lethargic and evidenced muscular weakness, continuous tremor, excess lacrimation, and incontinence of urine. These symptoms developed rapidly and rats became comatose and died, frequently within 6 to 8 hours after the first symptoms were observed. At necropsy[6] an acute hemorrhagic nephritis was invariably found.

In the series of experiments reported in this paper, this acute injury was avoided to a large extent by the method of feeding. A few rats showed mild symptoms but appeared to recover; in such cases evidence of old injury was apparent in the kidneys at necropsy after the completion of the experiment. Old lesions were also found in the kidneys of some animals which had never shown symptoms of injury.

With the exception of some diarrhea, the usual history in these experiments was uneventful during the first 4 or 5 weeks. On the basic diets supplemented with extract B there was very little growth; when these diets were supplemented with extract B-G considerable growth occurred. Following this there

[6] The authors express their appreciation to Mr. C. O. Prickett for the necropsy reports.

gradually developed the pigmentation and scaliness of the skin, the peculiar matted or ribbed appearance of the hair, alopecia, fissuring of the oral commissures, blepharitis, a variable edema and erythema of the feet, and evidence of nervous derangement as described by the above investigators. In addition, there has been rather constant occurrence of a profuse salivation, ulceration of the tongue, and a grayish spongy appearance of the buccal mucosa, in the later stages of the disease.

The skin lesions have been more severe and slightly different in character on rats receiving diets A and B than on those receiving diets C and D, or diets A and B supplemented with linseed oil. The rats on the low fat diets began to develop dry, rough skins about the third or fourth week. The epithelium exfoliated in small white scales which at first remained on the skin or were retained by the short, fur-like hair. Later there was desquamation of the epithelium in larger, thicker scales which would bring away tufts of hair. Deep fissures often appeared in the skin and from these blood exuded to form heavy brown incrustations. The scaly condition usually involved the feet, tail, and ears. The addition of linseed oil to diets A and B or the presence of 18 per cent of fat in other diets, had a pronounced effect in reducing the scaliness and fissuring of the skin. Instead of a dry condition, there was an excessive seborrheic secretion and a brown pigmented coating formed on the surface of the skin, particularly in the sacral and lumbar regions, when the fats were added to the diet; it was only in the last stages of the disease that any marked exfoliation of the epithelium occurred, the skin frequently remaining free from scaliness even after there was practically complete depilation. Some of the animals, however, produced severe traumatic lesions by biting or by lacerating the skin with the claws. The surface hemorrhages which occurred on the rats receiving the low fat diets were rarely seen on those receiving the medium fat diets; however, hemorrhages within the skin, which appeared as large brown pigmented areas, were not infrequent. On both types of diet

there usually appeared to be a congestion of the superficial blood vessels which gave the skin a peculiar pink color even in the early part of the experiment.

Gastric acidity and hemoglobin. It was frequently noted at necropsy that the stomach contents of rats showing a severe form of the egg white syndrome gave a negative Congo red test. Gastric samples were, therefore, obtained from a series of fifty rats which were receiving various egg white diets. The samples were taken following a 24-hour fast. Five milliliters of 1 per cent alcohol were then administered to each rat and 30 minutes later 3 ml. of H_2O were administered and as much as possible of the stomach contents aspirated immediately. The H-ion concentration of the specimens was determined by the quinhydrone method.

For rats not showing marked symptoms the gastric samples were usually within the range of pH 2.20 to 3.00. In the late stages of the disease, however, there was in most cases a significant decrease in the H-ion concentration, the range being from pH 4.00 to 6.50.

Hemoglobin determinations were made on the blood from sixty rats in various stages of the disease. Of these, twenty-three were well below normal values, being within the range of 10 to 12.5 gm. per 100 cc. There was apparently a tendency for the hemoglobin to decrease in the late stages of the disease. This tendency was less apparent on the low fat than on the medium fat diets.

Yeast and liver as supplements to the egg white diets. Table 3 shows the weights of rats which received dried brewer's yeast, dried baker's yeast, dried liver, liver extract, and the extracted residue of liver as supplements to basic egg white diets A and C.

The rate of gain was markedly increased and the occurrence of symptoms of the egg white disease was prevented by the feeding of 0.50 gm. of brewer's yeast per rat daily. The gains were much greater on diet C (group 13) than on diet A (group 12). The rats on diet A showed a dry, rough condition of the skin which was probably a mild form of the unsaturated fatty

acid deficiency disease. This was very mild, however, and was not to be compared to the miserable condition of the skin in groups 1, 2 and 3.

Baker's yeast (group 14) was much less effective than brewer's yeast, 0.50 gm. being little more effective in increasing the gain than a corresponding amount of extract B-G.

TABLE 3

Average weekly weights of rats. Raw dried egg white diets supplemented with brewer's yeast, baker's yeast, dried liver, liver residue, or liver extract

Group	12	13	14	15	16	17
Diet	A	C	C	C	C	C
Extract	—	—	—	B	B	B
Supplement[1]	Brewer's yeast	Brewer's yeast	Baker's yeast	Dried liver	Liver residue	Liver extract
Number of rats	6	4	6	4	4	4
Week	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Initial	48	53	51	53	54	54
1	48	80	51	72	74	69
2	68	108	71	96	91	79
3	92	135	92	107	106	88
4	113	154	108	107	116	92
5	130	176	119	124	127	102
6	144	193	127	136	134	115
7	157	211	137	148	142	128
8	168	223	145	158	151	134
9	172	234	149	167	158	141
10	180	248	156	174	165	146
11	188	264	158	178	168	148
12	—	—	162	182	173	150
13	—	—	—	189	176	155
14	—	—	—	194	181	160

[1] All supplements fed at rate of 0.50 gm. per rat daily except the liver extract which was fed at rate equivalent to 0.50 gm. of the extracted residue.

The rats receiving the baker's yeast developed the usual symptoms although probably in slightly less severe form.

Dried liver (group 15) was less effective than brewer's yeast in increasing the rate of gain but prevented all symptoms of the egg white disease. Liver residue (group 16) was only slightly less effective in promoting growth than the whole

dried liver, 0.50 gm. per rat daily preventing the occurrence of abnormal symptoms. Liver extract (group 17) equivalent to 0.50 gm. of the residue per rat daily was inferior to dried liver or liver residue in its effect on growth as well as in preventing the occurrence of abnormal symptoms. It had some protective value as the rats receiving the extract did not develop pronounced skin lesions. They did show, however, the short fur-like hair which was always seen as an early symptom.

Egg white + yeast residue and egg white + casein diets. The effect of adding yeast residue, hydrolyzed yeast residue, or extracted casein to the basic raw egg white diets is indicated by the weights of rats in table 4. The addition of 10 per cent of yeast residue to diet A produced a tremendous increase in the rate of gain (group 18). The yeast residue had an even more striking effect on the condition of the rats, group 18 appearing normal in every way except for a slight dry, roughened condition of the skin which was prevented by the addition of 0.20 ml. of linseed oil per rat daily (group 20).

The addition of 10 per cent of yeast residue to diet B, however, did not improve the rate of growth (group 22) nor did it prevent the occurrence of a severe form of the disease. The further addition of linseed oil improved the growth rate slightly (group 23) and effected a striking improvement in the condition of the skin. The addition of 20 per cent of yeast residue likewise failed to produce satisfactory growth (group 24) but the addition of linseed oil increased the growth rate (group 25); growth was still further improved by substituting extract B-G for extract B (group 26). Even in group 26 growth was very poor, although, with the exception of the subnormal rate of growth and a slight seborrheic condition of the skin, there were no apparent abnormal symptoms.

A comparison of group 27 with group 6 shows the response to the addition of 10 per cent of yeast residue to diet C. Growth was further improved by the substitution of extract B-G for extract B (group 28); group 28 made the best growth obtained in this series of experiments.

TABLE 4

Average weekly weights of rats. Raw egg white diets supplemented with yeast residue, hydrolyzed yeast residue, extracted casein, or white corn

Group	18	19	20[1]	21[1]	22	23[1]	24	25[1]	26[1]	27	28	29	30	31	32	33	34[2]
Diet	A-1	A-1	A-1	A-1	B-1	B-1	B-2	B-2	B-2	C-1	C-1	C-2	C-3	C-3	C-4	E	F
Extract	B	B-G	B	B-G	B	B	B	B	B-G	B	B-G	B-G	B	B-G	B-G	—	—
Number of rats	2	6	2	5	4	4	4	4	4	4	4	4	4	4	4	6	6
Week	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Initial	53	54	53	55	49	51	51	51	50	49	51	48	52	52	51	52	53
1	61	62	65	64	41[3]	45	51	56	59	52	81	50	53	78	62	60	65
2	81	87	89	88	47	57	53	63	68	62	111	69	56	102	68	72	83
3	107	113	112	112	54	65	58	68	79	71	138	73	66	121	118	83	98
4	126	138	130	133	60	75	59	73	87	85	160	88	79	136	137	93	119
5	141	158	153	152	63	79	63	80	94	108	183	100	83	149	150	101	141
6	149	177	166	169	60	79	64	81	98	138	210	108	88	144	159	112	160
7	160	182	179	184	61	79	66	88	106	158	230	121	90	139	164	123	170
8	165	200	190	197	69[3]	79	66	90	115	180	248	141	99[3]	135	172	133	184
9	182	210	196	208	68	82	67	93	119	197	262	153	102	131	183	144	196
10	191	222	216	221	64	85	65	97	126	204	281	161	102	—	184	150	200
11	199	231	222	231	63[3]	84	71	103	134	200	286	159	104	—	188	160	218
12	210	241	241	239	63	89	72	106	142	233	292	—	104	—	177	170	228
13	216	241	251	247	61	86	75	109	144	240	296	—	—	—	—	176	236
14	222	243	252	249	—	—	75	111	144	248	304	—	—	—	—	—	—

[1] Each rat in groups 20, 21, 23, 25 and 26 received 0.20 ml. of linseed oil per day.

[2] Group 34, on diet F, is included for comparison with group 33 on diet E.

[3] Indicates death of a rat.

Hydrolysis of the yeast residue largely destroyed its ability to increase growth and prevent the occurrence of symptoms (group 29).

Extracted casein at the 10 per cent level neither improved growth (groups 30 and 31) nor decreased the severity of the usual symptoms; at the 18 per cent level, there was some improvement in the growth of the rats but the syndrome still developed in severe form.

Group 33 received a diet containing 85 per cent of white corn and only 12 per cent of raw, dried egg white. Growth was poor and the usual symptoms developed. Group 34 received the same diet as group 33 except that extracted casein was substituted for the raw, dried egg white; although growth was subnormal in group 34, the rats were in good condition throughout the experiment.

Effect of various treatments of egg white. Cooking the dissolved, dried egg white for 30 minutes at 90 to 100°C. increased the rate of gain slightly (group 35, table 5) and decreased the severity of the symptoms; even so, the rats in group 35 were distinctly abnormal.

Raw, fresh egg white (group 36) was not superior to raw, dried egg white. In fact, symptoms developed earlier and appeared to be more severe when the raw, fresh form was fed.

Groups 37 and 38 received diet G containing fresh egg white which had been coagulated by heat and extracted with 51 per cent alcohol. For group 37 the diet was supplemented with extract B, providing a diet which was free from vitamin G. Consequently, these rats made practically no growth. They developed a mild conjunctivitis, ulceration of the tongue, and edema, erythema, and an exfoliating dermatitis of the feet; most of these rats showed a seborrheic condition of the skin, particularly in the sacral region, and a few developed a moderate amount of alopecia. For group 38 the diet was supplemented with extract B-G. The growth of these rats presents a striking contrast to the growth of those receiving a comparable diet containing either raw, fresh egg white

(group 36), raw, dried egg white (group 7), or cooked, dried egg white (group 35). The general condition of the animals in the various groups showed an even greater contrast, group 38 appearing normal in every way except the rate of growth which was below normal. Two of the rats in group 38 received 0.50 gm. of yeast residue per rat daily from the ninth

TABLE 5

Average weekly weights of rats. Diets containing cooked dried egg white, fresh raw egg white, or fresh egg white coagulated by heat and extracted; also egg white extract as source of vitamin G

Group	35	36	37	38	39
Diet	G	H	I	I	J
Extract	B	B	B	B-G	B[1]
Number of rats	4	5	6	6	4
Week	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Initial	50	63	51	52	50
1	50	81	56	73	54
2	59	80	56	89	70
3	64	92	57	107	69[3]
4	76	97	56	128	70
5	86	100	56	140	78
6	100	100	58	153	89
7	111	101	60	164	97
8	114		57	169	107
9	122		58	178	118
10	126		58	187	125
11	120		60	197	136
12	129		57	202	139[2]
13	134		57	209	160
14	136		53[3]	216	185

[1] Group 39 received egg white extract equivalent to 0.60 gm. egg white (dry basis) until the end of the fifth week when it was increased to 2.00 gm.

[2] At the end of the twelfth week extract B-G (equivalent to 0.50 gm. brewer's yeast) was given in addition to the egg white extract.

[3] Indicates death of a rat.

to the fourteenth week of the experiment. There was no evidence that this addition to the diet was beneficial.

Curative tests. A number of rats receiving diet C or H supplemented with extract B were used in curative tests. Rapid improvement resulted from the addition of 0.50 gm. of dried liver, brewer's yeast, yeast residue, or 5 to 10 ml.

of whole milk per rat daily. In most cases apparent cures resulted even though the rats were in miserable condition when the supplementary feeding was instituted. No improvement in condition followed the feeding of 0.50 gm. of extracted casein, gelatin, or of extract B-G equivalent to 0.50 gm. of brewer's yeast. Likewise, no response was obtained to the feeding of 0.20 ml. of dilute hydrochloric acid (1:10) per rat daily.

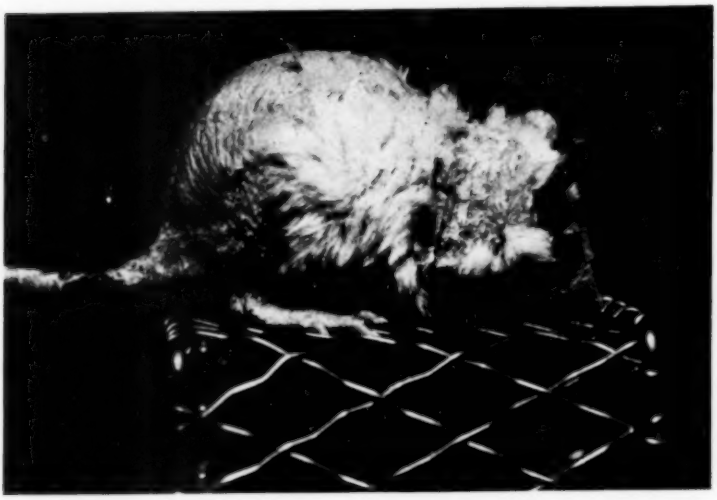

Fig. 1 Rat no. 4233, group 36, receiving diet H which contained raw, fresh egg white.

DISCUSSION

The data presented in this paper supplement the work of other investigators in showing that a peculiar physiological abnormality results when rats receive diets containing raw egg white as the sole source of protein. Such abnormality is not dependent upon an extremely high concentration of protein; it is readily produced on diets containing only 18 per cent (dry basis) of raw egg white. Nor is it necessarily associated with the use of egg white in the dried form; diets containing raw, fresh egg white produce just as disastrous results as those containing the dried material.

The most prominent symptoms produced by such diets are short, fur-like or woolly hair, alopecia, hyperemia, exfoliating dermatitis, skin hemorrhages, variable edema, and erythema with desquamation of the epithelium of the feet, conjunctivitis and blepharitis, salivation, ulceration of the tongue, spongy condition of the buccal mucosa, and a nervous disturbance which, in some cases, is apparently associated with a pruritus that causes the animals to bite or lacerate the skin. Diarrhea is frequently observed, limitation of food in the first week of the experiment being almost invariably necessary to control it. Diets containing only 18 per cent of dry egg white supplemented with yeast extract produce considerable growth in the early part of the experiment; some animals receiving such diets continue to grow slowly even after the appearance of marked symptoms of the disease but, eventually, growth ceases and the animals decline in weight and die.

The severity of the skin lesions is decreased and their character altered somewhat by the presence of a small amount of linseed oil or 18 per cent of butterfat or hydrogenated cottonseed oil in the diet. This is probably due to the correction of a deficiency of unsaturated fatty acids in the low fat diet rather than to any direct amelioration of the effect of the egg white. Even when the diets are supplemented with brewer's yeast which prevents the egg white disease, the skins of the rats receiving the low fat diets become dry and rough early in the experiment. This condition is mild, however, as compared to the condition produced by basic raw egg white diets which are low in fat. It is evident that with such diets the condition of the skin is affected by two distinct dietary factors.

Hypochlorhydria appears in the late stages of the disease but is seldom found before the other symptoms are well developed. Its late appearance does not indicate that it plays an important part in the causation of the disease. This is further indicated by the negative response of diseased animals to the administration of hydrochloric acid.

There is also a tendency for the hemoglobin concentration of the blood to decrease in the severe stages of the disease. This tendency is more apparent in the rats receiving the medium fat diets than in those receiving the low fat diets. It is probable that, in the latter, an anhydremia obscures any decrease in hemoglobin which may have occurred.

The abnormal symptoms are not produced by a diet which contains 18 per cent of egg white that has been coagulated by heat and extracted with 51 per cent alcohol. The symptoms are also prevented by the inclusion of such substances as liver, brewer's yeast, or the extracted residue of these substances in the raw egg white diet. They are not prevented by extracted casein, white corn, baker's yeast, extract of brewer's yeast, or the hydrolyzed residue of brewer's yeast. Liver extract is only partially preventive. Rats that have developed severe symptoms may be restored to an apparently normal condition in most cases by the feeding of brewer's yeast, extracted residue of brewer's yeast, dried liver, or milk; they cannot be restored by the feeding of extracted casein, gelatin, extract of brewer's yeast, or dilute hydrochloric acid.

It is possible that the discrepancy in the findings of Boas and of Parsons regarding the potency of yeast may be explained by differences in the yeasts used. Parsons apparently used a baker's yeast grown on a synthetic medium. In the experiments reported in this paper a baker's yeast had a very low potency, if it could be considered at all protective. On the other hand, a full grain-grown brewer's yeast was very effective, comparing favorably with beef liver.

The egg white syndrome presents features which are suggestive of a deficiency disease. However, the demonstration that the diet is innocuous if the egg white is coagulated and extracted (or, as Parsons has recently shown, if it is merely cooked) instead of fed raw, shows that, if a deficiency is present, it is due to poor digestion of the raw protein rather than to its composition. Bateman ('16) and others have shown that raw egg white is not well digested and that its presence

even inhibits the digestion of other proteins. The fact that the syndrome is produced by the addition of 18 per cent of raw egg white to a casein diet which alone would permit normal growth may possibly be due to the latter effect of the raw egg white. Nevertheless, it does not seem that the presence of raw egg white should affect the digestion of casein or gelatin more than that of brewer's yeast, liver, or the other proteins of milk. Moreover, animals tend to develop a tolerance to raw egg white which enables them to digest it more efficiently (Bateman, '16).

On the other hand, the typical syndrome develops only after the rats have received the raw egg white diets for several weeks, during which time considerable growth may have occurred. In this condition there certainly is no evidence of the development of a tolerance. The results may be explained on the basis of positive injury by the raw egg white more readily than on the basis of a deficiency. If this is the correct assumption, then it follows that the harmful factor in the raw egg white is antagonized by the substances which have been shown to be protective.

The symptoms of egg white injury are sufficiently similar to those produced by a deficiency of vitamin G to indicate the possibility that the harmful factor, if present, has an anti-vitamin G action. Since increasing the supply of vitamin G does not prevent the symptoms and since the vitamin G free residue of brewer's yeast or liver does prevent them, it is apparent that the theory of an anti-vitamin G action is untenable. Although increasing the supply of vitamin G in the egg white diets by the addition of extract B-G does not prevent the onset of symptoms, it increases the rate of growth. The vitamin G fraction of yeast extract apparently corrects a deficiency in the vitamin G fraction of the egg white. Further evidence of this supplemental effect is seen in group 39 where a marked stimulation of growth resulted from the use of extract B-G in addition to the extract from coagulated egg white.

The similarity of the symptoms in egg white disease, vitamin G deficiency, and pellagra is peculiarly interesting if one accepts the theory that native egg white contains a positive substance which is capable of producing such symptoms. It is evident that vitamin G deficiency is not the only condition in which pellagra-like symptoms may occur. The pigmentation and the dermatitis in egg white disease are even more typical of pellagra than are the symptoms of vitamin G deficiency. Numerous investigators have commented upon the variable character of the dermatitis in vitamin G deficiency. In this laboratory it has been observed that if a fuller's earth adsorbate obtained from extracts of brewer's yeast is used as a source of vitamin B in vitamin G deficient diets for rats, there is no extensive loss of hair, and generalized skin lesions are rarely seen; the only prominent symptoms are conjunctivitis, ulceration of the tongue, dermatitis in the nasal and oral regions, and edema, erythema, and exfoliation of epithelium of the feet. However, if the vitamin B adsorbate is prepared from white corn there is marked alopecia, and generalized skin lesions are frequent and severe. The experiments have not indicated whether the differences are due to other nutritive factors carried by the vitamin B preparation from brewer's yeast or to the presence of injurious substances in the preparation from corn.

SUMMARY

The use of raw egg white as the sole dietary source of protein for rats produced fur-like or woolly hair, alopecia, exfoliating dermatitis, hyperemia, skin hemorrhages, blepharitis, stomatitis, salivation, variable edema and erythema of the feet, symptoms of nervous disturbance, and finally hypochlorhydria and some anemia.

The results obtained from fresh egg white and dried egg white were similar when both were fed in the raw form.

The use of extremely high concentrations of protein was not essential to the production of the disease; diets containing only 18 per cent of raw egg white (dry basis) caused severe symptoms.

The skin lesions were more severe on low fat diets than on diets containing 18 per cent of butterfat or hydrogenated cottonseed oil or 0.20 ml. of linseed oil per rat daily.

If the diet contained not more than 18 per cent of raw egg white (dry basis) the symptoms were prevented by the inclusion of brewer's yeast, dried liver, or the extracted residue of brewer's yeast or liver in the diet but not by the inclusion of extracted casein, extract or hydrolyzed residue of brewer's yeast, white corn, or baker's yeast; liver extract was more effective than yeast extract but was only partially preventive.

Well developed cases of the disease were apparently cured by brewer's yeast, dried liver, milk, or extracted residue of brewer's yeast but were not improved by extracted casein, gelatin, extract of brewer's yeast, or dilute hydrochloric acid.

Coagulation of fresh egg white by heat and extraction of the coagulum with 51 per cent alcohol rendered it innocuous.

The concentrated extract of egg white had a slight harmful effect but failed to produce the severe symptoms caused by raw egg white. This extract, as a source of vitamin G, was less effective than an extract of brewer's yeast.

The data indicate a positive harmful factor in raw egg white which is antagonized by the protective substances rather than the existence of a deficiency.

The results are apparently not due to an anti-vitamin G action of the harmful factor, although many of the symptoms are similar to those produced on certain vitamin G deficient diets and even more similar to those of pellagra.

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STORAGE OF VITAMIN A IN CATTLE [1]

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ONE FIGURE

(Received for publication October 23, 1933)

In previous publications (Hart et al., '32, '33a, '33b) we have shown that cattle on the range in California are subject to low vitamin A intake during the dry season. The length of the drought period varies from year to year, with a corresponding variation in the manifestations of vitamin A deficiency. Under natural conditions a single deficiency uncomplicated by other factors rarely occurs. This fact, coupled with the variation in the syndrome of vitamin A deficiency, rendered observations difficult to interpret without fuller knowledge of mild evidences and of the complications that arise as the symptoms become more acute, finally ending in death of the animals.

The three most important manifestations of vitamin A deficiency in cattle are expulsion of the fetus prematurely or dead at term, severe diarrhea in newborn calves, and ophthalmia in young growing animals. These conditions are easily confused with bovine infectious abortion, white scours in calves, and infectious keratitis. The differential diagnosis of these conditions has been described by us (Hart and Guilbert, '33 b).

[1] This report is part of an investigation on the relation of nutrition to reproduction in livestock, which became cooperative with the United States Bureau of Animal Industry, July 1, 1929.

The ability of cattle to store vitamin A in times of abundance against periods of privation is extremely important under range conditions in California. It is valuable therefore to have some knowledge of the extent of this reserve in order that supplements may be most economically supplied to prevent deficiency.

Halverson and Sherwood ('30) have definitely demonstrated that the so-called cottonseed poisoning in cattle is primarily caused by the vitamin A deficiency of rations consisting of cottonseed hulls and meal or combinations of these feeds with white corn, oats or beet pulp.

Experience in commercial feed lots shows that heavy feeding of steers on cottonseed meal and hulls for periods in excess of 100 days is liable to cause blindness, loss of appetite, and rapid loss of weight in many animals. A difference in the time of encountering these symptoms, depending on whether or not the steers are brought into the feed lot from good feed (which usually means green feed) or poor feed, is also recognized. In one experiment Halverson and Sherwood ('30) report the appearance of various eye lesions and of blindness without visible lesions, in a large percentage of steers 88 days after being fed in dry lot on an exclusive ration of cotton-seed hulls and meal. These authors do not mention the previous feeding of these animals. According to other experiments which they report, dairy heifers averaging less than a year of age may subsist on such a ration for 200 days before the onset of acute symptoms. In nearly all cases, however, death ensued soon thereafter unless the ration was changed.

Mead and Regan ('31) reported the development of avitaminosis A in calves 1 to 3 months after being changed from whole milk and grain to a concentrate mixture low in vitamin A. No roughage was fed.

These and other data indicate variability in the onset of deficiency symptoms, depending on stage of maturity, lactation, previous feed supply and degree of deficiency of the rations.

This paper primarily considers the time required to deplete non-lactating cattle that have had ample opportunity to store vitamin A. The animals used were beef steers and heifers continuously supplied with green feed until placed on experiment. The storage of vitamin A in livers was studied by means of the blue color reaction with antimony trichloride.

EXPERIMENTAL

Technic used in color test for vitamin A. The use of the Carr-Price ('26) reaction for quantitative estimation of vitamin A offers increased opportunity to extend knowledge on vitamin A problems in the field of livestock production, even though it may have limitations as a precise method of vitamin A assay. The examination of the vitamin A content of liver tissue by this method is an excellent diagnostic aid in cases of suspected natural occurrence of the deficiency, especially in borderline cases where definite clinical symptoms are lacking.

It is recognized that a number of substances may react with antimony trichloride to form a blue color. In a rather wide experience, however, we have obtained at most a trace of blue color; more commonly none from the liver tissue of animals known to be depleted of vitamin A. On the other hand, we have never failed to obtain strong reactions with extracts of liver tissue from animals which were abundantly supplied.

In sampling liver tissue of large animals, two slices are generally taken from different parts of the organ. We have found no difference in the various cross sections of the liver. Thin peripheral slices, however, have proved less potent in vitamin A than sections nearer the center. The samples are ground in a food chopper and mixed; then duplicate 10 to 20 gm. samples are weighed into 250 cc. Erlenmeyer flasks.

The digestion and extraction of liver tissue closely follows the method of Moore ('30). Twenty to forty cubic centimeters of 5 per cent KOH are poured into the flask containing the liver tissue, which is then brought into solution by heating for a few minutes in a boiling water bath. The strength

of KOH is of minor importance; we obtain the same results with 5, 20 and 50 per cent KOH. The stronger alkali solutions are used when complete saponification is desired.

After the liver tissue has been brought into solution and cooled, the contents of the flask are transferred to a separatory funnel, and 20 to 30 cc. of 50 per cent ethyl alcohol added. The solution is extracted twice with ethyl ether, using a total of three to four volumes compared with the solution to be extracted. After the alkaline layer has been drawn off, the ether solution is washed free from alkali with distilled water, and is transferred to a flask containing 5 to 10 gm. of anhydrous sodium sulfate. After a vigorous shaking it is allowed to stand for a few minutes. The solution is then decanted, and the sodium sulfate washed twice with small volumes of fresh ether. Next the ether is distilled off, care being taken thereafter not to subject the residual material to heat. The flask containing the liver oil (or only the unsaponifiable fraction) is then placed in a vacuum desiccator. If the material is to stand for some time under vacuum, the air is first washed out with CO_2. A few minutes under vacuum, however, suffice to evaporate remaining traces of ether, the water having been largely removed by the previous contact with anhydrous sodium sulfate. The residue is dissolved in chloroform to a suitable volume. If the liver oil in question is suspected of being low in vitamin A, we use as small a volume as possible (1 to 2 cc.). For normal liver tissue a dilution with chloroform to 10 cc. is satisfactory. Since the intensity of the blue color formed with antimony trichloride depends both upon the concentration of the vitamin or precursors and upon the concentration of the reagent, the test solution must be kept to small volume. The test should be conducted as soon as possible after the material is dissolved in chloroform for the vitamin appears to be unstable in chloroform solution. We have found potent samples to have changed in standing overnight to such an extent that only the red or reddish-brown color developed.

Quantitative estimations are made by diluting the test solution until 0.05 to 0.2 cc. with 2 cc. of antimony trichloride reagent produces a color that approaches the vanishing point. The quantity of the solution required may then be calculated back to the amount of liver tissue it represents.

In our work a unit is defined as the amount of chromogen in 0.05 to 0.2 cc. of chloroform solution which will give the faintest detectable blue color with 2 cc. of antimony trichloride reagent. We have found that 1 to 1.5 gamma of crystalline carotene (S.M.A. Corporation. M.P. 166° to 168°) is equivalent to 1 color unit. If 0.5 gamma of carotene is considered 1 rat unit, then 1 color unit is approximately 2.5 R.U. Since vitamin A produces ten to twenty times as much color with antimony trichloride as carotene (Moore, '33) and since he has further shown that vitamin A and carotene are equal biologically, 1 color unit in terms of vitamin A would be equivalent to 0.1 to 0.25 R.U. Our unit should not therefore be confused with blue units based upon the Lovibond tintometer.

Reasonably good agreement between duplicate samples among three independent workers in this laboratory has been obtained by this method. Since adopting this technic, we have noted a paper by Andersen and Nightingale ('29), who employed a similar dilution procedure for quantitative tests for vitamin A in butter, margarine and other fatty foods.

In order that judgment of the end point may not vary too much because of light variations, comparison is made against a solution of $CuSO_4$ equivalent to 0.5 mg. of copper per cubic centimeter, kept in a sealed tube of the same diameter as that used for the test solutions. The antimony trichloride reagent used is a saturated solution in C.P. chloroform at room temperature, the crystals having first been washed with a little chloroform. About 30 gm. of antimony trichloride to 100 cc. of chloroform are required.

Animals and rations used. Twelve beef steers, varying in age from 9 to 20 months, were selected in September, 1932, from the University beef herd. All these animals had grazed

on native pasture during the previous spring. When the grass dried they were either pastured on irrigated alfalfa or sudan grass or fed in dry lot on a ration consisting of concentrates (barley, oats and wheat bran) fresh green corn, alfalfa and sudan grass hay until placed in the experiment. The conditions may therefore be considered ideal for storage of vitamin A. Four animals constituting group 1 were killed at the beginning, and samples of the livers were obtained. Six head, constituting group 2, were placed on a low vitamin A ration consisting of dried molasses beet pulp 70 per cent, rolled barley 14 per cent, cottonseed meal 15 per cent, and $CaCO_3$ 1 per cent. The two remaining animals, constituting group 3, were fed the same grain ration as those in group 2, but each received in addition 1 pound daily of high-quality field-cured alfalfa hay. The average amount of feed consumed was 15 pounds of the concentrate mixture daily. No apparent difficulty was encountered from lack of roughage in the ration. The steers in groups 2 and 3 were killed after varying intervals on these rations and the livers were examined for their content of vitamin A. The first pair from group 2, nos. 427 and 441, were killed after 63 days; the second pair, nos. 424 and 445, after 121 days, and the third pair, nos. 444 and 428, after 282 days, in an advanced stage of vitamin A deficiency. Steers 422 and 425 of group 3 were autopsied after 127 days.

The data obtained from the liver samples of these animals are presented in table 1.

Table 1 shows the rather uniform storage in the livers of the group 1 animals and the progressive depletion of the reserves in the animals of group 2 as the time on the deficient diet advanced. The feeding of 1 pound of high quality alfalfa hay to group 3 apparently had little effect upon the maintenance of reserve in their livers. We have some evidence, however, that this quantity suffices to delay the appearance of clinical symptoms and may be valuable under certain conditions in supplementing vitamin A deficient range.

The onset and progress of clinical symptoms of vitamin A deficiency in steers 428 and 444 of group 2 were as follows:

On June 7th, 225 days after being placed on the experiment, no. 444 showed excessive lacrimation, especially in bright sunlight, and on June 30th showed night blindness for the first

TABLE 1

Depletion in storage of vitamin A in the livers of steers fed a ration deficient in this essential

GROUP NO.	STEER NO.	AGE WHEN SLAUGHTERED	BREED	INITIAL WEIGHT	FINAL WEIGHT	FEEDING PRIOR TO SLAUGHTER	UNITS PER GRAM OF LIVER[1]
		months					
	419	17	Crossbred	....	1050	Grain, green fodder and hay	500
I	421	17	Hereford	....	925	Green pasture	500
	437	12	Hereford	....	820	Green pasture	500
	417	19	Shorthorn	....	800	Grain, green fodder and hay	830
	427	20	Shorthorn	1012	1100	63 days on ra-	250
	441	13	Aberdeen Angus	765	845	tion deficient in vitamin A	420
	424	21	Shorthorn	1020	1165	121 days on ra-	160
II	445	13	Shorthorn	600	800	tion deficient in vitamin A	50
	444	19	Shorthorn	630	1050	282 days on ra-	Trace†
	428	25	Shorthorn	1020	1280	tion deficient in vitamin A	Trace†
	422	20	Crossbred	1080	1195	127 days on ra- tion deficient	80
III	425	20	Crossbred	955	1090	in vitamin A + 1 pound alfalfa daily	50

[1] See definition of units in section on technic.

time. He manifested temporary blindness when driven around the corral in semi-darkness. On June 23rd, no. 428 had a slight nasal discharge, and his nose was dry and scaly. On July 4th, he exhibited night blindness and muscular incoordination. On July 7th, when the animals were tested in semi-darkness, both steers ran into the fence and posts in the

corral repeatedly, although normal animals had no difficulty in avoiding them. Finally, the steers came together head on; and, although the force of the impact was not great, no. 428 dropped as though felled by a stunning sledge, rolled to his back, and quivered with his legs extended. After a few seconds he was able to regain his feet.

From this time on, sight became more and more defective until there was considerable impairment even in daylight. The degree of impairment varied from one time to another, as did also the muscular incoordination, which took the form of staggering from side to side when these steers were driven about the lot. At times the rear legs appeared most affected. On July 12th, both steers were discharging mucus from their nostrils continuously. Tears flowed from their eyes, keeping the hair wet down the sides of their faces. Aside from slight inflammation and edema of the nictitans membranes of steer 444, no visible eye lesions were noted. The corneas did not become clouded in these cases.

On July 15th, the nose of steer 444 appeared slightly twisted laterally, suggesting partial facial paralysis. On July 26th, steer 428 showed definite facial paralysis: the left ear drooped and flopped when he moved about, and the left eye appeared somewhat sunken. During this time appetite remained good, the steers regularly consuming their daily allowance of 15 pounds of concentrate mixture. On July 29th, however, a mild diarrhea of intermittent nature appeared, and part of the feed was not eaten. Up to this point the general appearance of the animals had been good, but in the next few days the decline was rapid. The nasal discharge, sight impairment, and muscular incoordination became more marked. The hair began to appear rough, appetite failed, and weight rapidly declined. On August 4th the steers were slaughtered after 282 days on the deficient ration.

On post mortem examination the internal organs appeared normal. Both steers had a dressing percentage of about 65, indicating a high degree of fatness. The fat was pure white, whereas the fat of those killed at 60 to 120 days was a creamy

yellow, and that of steers killed at the beginning of the experiment carried considerable pigment. This experiment shows that carotene, the principal pigment of beef fat, may be withdrawn from the adipose tissue during vitamin A privation without a coincident reduction of the fat reserve. The carotene in the fat of cattle undoubtedly constitutes a significant part of the total vitamin A reserve. All these animals were fat enough for slaughter when placed on the experiment and consequently may have had more reserve than thin animals on the same kind of feed.

With antimony trichloride reagent the non-saponifiable matter from 20-gm. samples of the livers of these steers gave a faint violet color, probably caused by a trace of blue color combined with the red commonly encountered when the extract is devoid of vitamin A.

The weight curves of these steers are presented in figure 1. These show nearly uniform weight increases at the rates of 1 and 1.3 pounds daily, respectively, up to the time when vitamin A reserves were practically exhausted and the appetites of the animals had failed.

Storage of vitamin A in heifers. This was an experiment to study the effects of nutritional deficiencies during gestation. The basal ration used consisted of dried molasses beet pulp, wheat straw, and calcium carbonate; it was definitely deficient in protein, phosphorus, and vitamin A. Heifers 15 to 20 months of age, free from infectious abortion, were placed on experiment soon after becoming pregnant. Two of them, nos. 426 and G 13, were fed the basal ration. The former which came from the University beef herd had abundant vitamin A in her ration until placed on experiment. After being on this ration for about 8 months, she sustained marked loss in weight, declined to low levels in blood phosphorus, and manifested marked osteophagia and other forms of pica. She produced at term a live but undersized calf and developed no clinical evidence of vitamin A deficiency. Because her milk supply was meager, growth of the calf was extremely limited.

The calf lived for 5 months upon this meager milk supply supplemented by straw and concentrate mixture eaten with the mother. Its weight increased only 25 pounds throughout the period. While general under-nutrition contributed to the death of this calf, definite symptoms of vitamin A deficiency were recorded at post mortem. The crystalline lenses of both eyes were clouded, although the corneas were not affected.

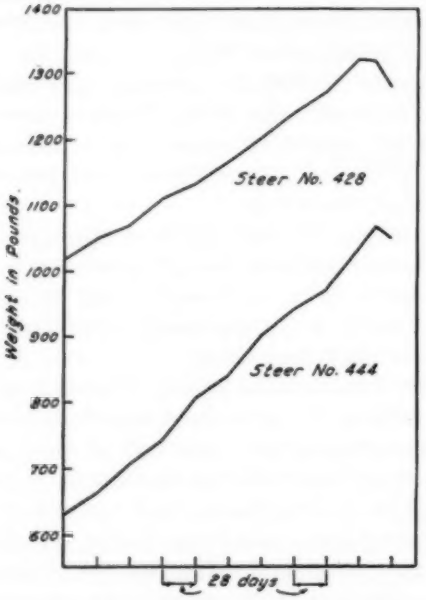

Fig. 1 Weight curves of steers 428 and 444, showing continuous gains until the time when vitamin A reserves were practically exhausted and the appetites of the animals had failed.

The right lung had pneumonic areas in which were nodules containing air and pus cells similar to other cases examined involving vitamin A deficiency (Hart and Guilbert, '33 b). The extract from 20 gm. of the liver tissue, concentrated to small volume, gave no test for vitamin A. Mild diarrhea had been noted at intervals prior to death. It is interesting that

the dam of this calf has continued for 14 months without showing symptoms of vitamin A deficiency. We venture the explanation that the multiple deficiencies of the diet resulting in the animal bordering on starvation greatly retarded the depletion of reserves as compared to the steers of comparable age on a more complete ration.

The second heifer, no. G 13, was range raised and was purchased in the fall after having been on dry forage during the summer. She was placed on pasture containing some green feed and also received hay for 6 weeks before going on the basal ration. This heifer exhibited marked night blindness after 195 days. She aborted the two hundred and twenty-ninth day of pregnancy, after being on the ration 205 days. Upon slaughtering, her fat was found to be white, and extensive abscess formation was present in one lung. A sample of her liver gave a color test of only 2 units per gram. The liver tissue of the fetus gave no color test for vitamin A.

Heifer 429, from the University herd was fed 1 pound of high-quality field-cured alfalfa hay in addition to the basal ration. She carried her calf to term (283 days) but parturition was difficult. The cow was found exhausted in labor in the morning with the fetus anteriorly presented in the pelvic cavity. Strong traction was necessary for its delivery. It was a large calf weighing 83 pounds and probably died during parturition. Its liver extract showed a trace of blue color with antimony trichloride.

Four other heifers calved normally at term. The diets of two of them, nos. G54 and 440, were modified to eliminate phosphorus deficiency and in the other two, nos. 410 and 440, to eliminate phosphorus and protein deficiency; but in all cases practically the sole source of vitamin A was 1 pound of alfalfa hay daily.

The calves from nos. G54 and 410 were normal until 7 or 8 days of age when they developed a diarrhea, which became so severe that white-colored liquid feces were discharged at frequent intervals. Suspecting that inadequacy of vitamin A might be involved, cod liver oil of high biological value was

given by mouth in 10 cc. doses daily to the calf from no. G54 which was most severely affected. The other calf was kept as a control. Improvement in the fecal discharges and physical appearance of the calf receiving cod liver oil was evident by the fourth day, whereas the control calf was rapidly becoming worse. Cod liver oil was then administered in the same dose and improvement followed, both calves returning to normal in 8 or 9 days when the oil was discontinued. The calves grew at nearly normal rate for 2 months, at the end of which time the calf from no. G54 showed marked night blindness and again developed severe diarrhea. A day or two later the calf from no. 410 also had diarrhea. The latter was again kept as a control and the former treated with cod liver oil. The night blindness and diarrhea disappeared after 4 days. The control calf continued to have diarrhea for several days, then recovered without treatment. For 10 days prior to the second onset of diarrhea these calves were given 1 pound each of dried molasses beet pulp. Since they were fed together it is possible that the G54 calf may have eaten less than her share after the onset of diarrhea and overeating may have contributed to the diarrhea in the calf kept as a control.

Heifer no. 438 had an apparently normal calf which weighed 73 pounds at birth. This calf developed severe diarrhea the third day following parturition and was given 10 cc. daily of cod liver oil. It declined rapidly, however, developed pneumonia and died 3 days later. Post mortem examination showed mild general enteritis and broncho-pneumonic areas in the left lung. The antimony trichloride test for vitamin A in the liver gave a relatively high value of 125 units per gram, presumably because of the cod liver oil feeding.

Heifer no. 440 calved normally. The calf weighed 82 pounds but appeared weak. After being assisted to its feet the calf nursed, but on the second day developed severe diarrhea. The appetite declined and it nursed very little. No treatment was given. After 5 days improvement was noted, appetite returned and within the next week it grew active and strong.

The mother was milked out regularly during the period when the calf did not take all the milk, as was also done with heifer no. 438.

Milk samples were taken from these heifers at varying times and the antimony trichloride test was applied to the unsaponifiable fraction of the milk fat. The results are presented in table 2. The first samples from nos. G54 and 410 were taken at the time their calves developed diarrhea. The apparently negative result obtained for no. 410 must be con-

TABLE 2

Antimony trichloride color test on milk fats

DESCRIPTION OF SAMPLE	UNITS PER GRAM OF MILK FAT				
	No. G54	No. 410	No. 438	No. 440	Dairy herd
Colostrum, 1st day				360	
Colostrum, 2nd day				155	
Colostrum, 3rd day			150	120	
Milk, 7th day			20		
Milk, 10th day	Negative	?	10		
Milk, 14th day			20	7	
Milk, 24th day			15		
Milk, 43rd day				14	
Milk, 3½ months	Trace	7			
Milk, 4 months	Negative	Trace			
Milk, 5 months	Negative	Negative			
Composite sample—winter milk					27
Composite sample—winter milk					33
Composite sample—winter milk					35

sidered questionable since the butter fat content of the milk was very low and some difficulty was encountered with this sample. All other results have been run under conditions known to give consistent results on normal milk. The fact that these animals were not accustomed to being milked and had to be restrained discouraged us from taking samples frequently in the early part of the experiment.

Heifer G54 was range raised and had the same previous history as heifer G13 which developed clinical symptoms of vitamin A deficiency and aborted. The fact that the former animal received 1 pound of alfalfa hay daily containing ap-

proximately 16 mg. of carotene possibly explains the normal completion of her gestation. This amount did not, however, provide sufficient for her to secrete detectable quantities in the milk, nor to prevent the onset of symptoms of vitamin A deficiency (night blindness) in the calf at about 2½ months of age, even after it had been given 10 cc. daily of cod liver oil from the eleventh to the twentieth day after birth.

The other three heifers were from the University herd and had ample opportunity to store the maximum vitamin A for their age, prior to the experimental period. Table 2 shows that after the colostrum period, their milk fat contained less than half the vitamin A found in winter milk samples from the dairy herd. The butter fat of the experimental heifers was practically colorless, while the dairy herd samples contained considerable pigment. Since carotene gives much less blue color with antimony trichloride in relation to its biological value than does vitamin A, the difference between the samples shown in table 2 is minimized.

None of these heifers, except G13, have shown clinical evidence of vitamin A deficiency up to 6 months after parturition.

The relation between occurrence of diarrhea in the calves to the inadequacy of the diet of the dams is not as yet clear. The regularity of its occurrence appears significant as well as the recurrence of the disorder simultaneously with night blindness and the apparent response to vitamin A therapy in the case of the calf from no. G54. Since, as will be shown in a later section of this paper, the reserves at birth are low even in calves from cows having abundant storage, and since the liver of one calf that died during parturition in this experiment had only a trace of vitamin A, it is possible that the calves in utero received an inadequate supply, even though the colostrum of at least two of the dams was found to be comparatively rich in the vitamin. The relating of diarrhea to vitamin A deficiency by other investigators and our own observations in the naturally occurring cases on the range support this view.

Storage of vitamin A in the livers of mature cows. Liver samples were obtained from two aged cows from the University beef cattle herd. These animals were 11 and 12 years old, respectively, and throughout life had access to green pasture for at least 9 months of every year. During the remainder of the year they received a ration of alfalfa hay and silage. Both cows had produced calves regularly. No. 63 was practically dry at the time of slaughter, having nursed a calf for a period of about 8 months. No. 75 had not been producing milk for several months prior to slaughter. Both cows were excessively fat.

Ten additional samples of liver were obtained at a local slaughter house from a lot of cows that had been for 7 months on range feeds deficient in vitamin A. During the early part of the dry feed period the cattle grazed native pasture. Later they were pastured on barley stubble and during the last 6 weeks before slaughter were fed in addition to dry pasturage, 10 pounds of barley and a small allowance of sesame meal daily. These cows were all well along in years and were not lactating. Most of them probably had not produced calves the previous spring. The fat on the carcasses of these animals contained considerable yellow pigment.

The results of the tests on the livers are presented in table 3. Cows nos. 63 and 75, from the University herd, had a concentration of vitamin A in their liver tissues approximating ten times that found in the growing steers 12 to 20 months of age. The liver tissues of these cows were comparable in concentration of vitamin A to values found for high potency cod liver oil. The non-saponifiable fractions of these livers are therefore an exceedingly concentrated source of vitamin A. The non-saponifiable matter was fractioned by the phase method. As shown in table 3, nearly all of the chromogenic value was found in the 85 per cent methyl alcohol fraction. Practically all of the yellow pigment, however, remained in the petroleum ether fraction, showing it to be carotene. Since the methyl alcohol fraction was nearly colorless, vitamin A must have been responsible for practically all of the blue color formed by the reaction with antimony trichloride.

The liver storage in the range cows that were killed after 7 months on dry feed varied widely. The highest storage was only one-fifth that found in the University cows. The four samples that were lowest in vitamin A (80 to 200 units per gram) indicate storage sufficient to protect the animals for several months if the experience with the steers previously cited may be taken as a criterion. Since these cows were mature and had not been lactating for some time, it is reasonable to expect slow depletion of reserve. The varia-

TABLE 3

Storage of vitamin A in the livers of mature cows

DESIGNATION OF SAMPLE		UNITS PER GRAM OF LIVER		
		Total (direct determination)	Petrol ether fraction	85 per cent methyl alcohol fraction
No. 63	University herd	4500	65	4400
No. 75	University herd	5000	80	5000
No. 1	Range cow	800		
No. 2	Range cow	500		
No. 3	Range cow	550		
No. 4	Range cow	500		
No. 5	Range cow	80		
No. 6	Range cow	200		
No. 7	Range cow	125		
No. 8	Range cow	400		
No. 9	Range cow	80		
No. 10	Range cow	1000		

tions found may be related to past history of the animals of which we have no record.

Storage of vitamin A in the livers of calves. Busson and Simmonet ('32) have reported the livers of newborn pups to be low in vitamin A even when the maternal liver contained large amount. Dann ('32) has also presented data showing the livers of rats and rabbits to be relatively low at birth, the storage being increased during the nursing period when the mothers were fed diets rich in carotenoids.

The livers of several calves have been tested for their content of vitamin A. The mothers, except the one noted in

table 4, had been on rations containing abundant vitamin A (good quality alfalfa hay, silage and pasture). The results are presented in table 4.

Since we have obtained values as high as 5000 units per gram from liver samples of older animals, it is evident from the data in table 4 that the storage in the liver of newborn calves is relatively low regardless of the storage in the dam. The liver extract of calves is practically colorless, and presumably the chromogenic substance is preformed vitamin A.

TABLE 4

Vitamin A content of the livers of calves

DESCRIPTION OF CALF	UNITS PER GRAM OF LIVER
Shorthorn born dead. Mechanical difficulties at parturition	42
Hereford, twin calves. Died at birth 10 days premature	12
Jersey. Killed 18 hours after birth. Had mother's milk	14
Jersey. Killed 36 hours after birth. Had mother's milk	10
Jersey. Killed 3 days after birth. Had mother's milk	5
Liver from cow receiving 75 cc. cod liver oil daily as sole source of vitamin A.	250
Liver of 7½ months' fetus from above cow	100
Holstein calf. Born dead	25

In normally-fed bovines, on the other hand, the liver extracts carry considerable carotene. These results agree with those of Palmer ('22), who found that tissues of newborn calves were practically free from carotenoids.

DISCUSSION

The data presented in this paper show that there is a variation in the time in which vitamin A deficiency may be manifested in animals on a deficient diet. The principal factors are storage reserves and the production requirements of the animals. Lactation is probably a greater strain on reserve supply than is gestation. During the period of active growth there is also a relatively greater requirement and less storage than when adult size is attained. The low reserves in the liver of newborn calves and other animals is evidence that the

pregnant mother is not able to mobilize this essential in the tissues of the fetus much faster than it is utilized. Evidently, therefore, the fetus will be the first to suffer when the borderline of deficiency is reached. Drummond, Coward and Watson ('21) showed that colostrum has a higher biological value and is richer in carotenoids than later milk from the same animal—a fact also shown by our chemical determinations. This is a very interesting parallelism to the concentration of euglobulin, carrying immune bodies in this important physiological secretion for the newborn, while, on the other hand, iron, which can readily pass to the fetus in utero, is stored in excess against a low intake during the milk-drinking period. Mead's (unpublished data) studies on the raising of dairy calves at this station have brought to our attention the importance of colostrum from the vitamin A standpoint.

Animals on the range that are able to obtain only a submaintenance intake of feed, utilize vitamin A less rapidly than animals on a supermaintenance and otherwise complete diet. Hence night blindness, ophthalmia and other acute evidences of deficiency are more liable to be found where range feed is being supplemented with vitamin A deficient concentrates than on range feed alone.

The storage of vitamin A during the green feed season is sufficient to carry range animals safely through dry-feed periods of ordinary duration. When, however, early drying of forage in the spring is coupled with late rains in the fall, and especially when two such seasons occur in succession, reserves may be completely depleted and clinical symptoms appear.

In supplementing the range, one must consider the vitamin A reserve of the animals and the length of the deficiency period in order to meet all the deficiencies. Cottonseed cake, ideal as a protein and phosphorus supplement, must itself be supplemented with alfalfa or other sources of vitamin A when this deficiency appears. Early recognition of mild evidence of vitamin A deficiency makes it possible to avoid serious loss.

SUMMARY

Clinical symptoms of vitamin A deficiency were evident in two beef steers after 225 to 240 days on a ration of dried molasses beet pulp, rolled barley, cottonseed meal and calcium carbonate. Reserves were practically exhausted, and the animals were in critical condition after 282 days, at which time they were autopsied. The gradual depletion of reserves with advancing time was followed by estimating the vitamin A potency of the liver oil of steers autopsied after varying periods on the deficient ration, the Carr-Price color reaction being used. According to evidence presented, the carotene in the adipose tissue, which constitutes a part of the vitamin A reserve, may be withdrawn during vitamin A privation without coincident withdrawal of the depot fat. All the steers used had ample opportunity to store vitamin A before the experimental period.

The expulsion of the fetus before it is viable, in the absence of infectious abortion, is described. Calves from heifers having a restricted intake of vitamin A during gestation developed a severe diarrhea at 2 to 8 days of age. The milk of the dams was shown to be deficient or subnormal in vitamin A. One calf exhibited marked night blindness although no clinical evidence of vitamin A deficiency has appeared in the dams up to 6 months following parturition. The liver tissue of mature beef cows, reared under favorable conditions, was found to have a concentration of vitamin A approximating that of high potency cod liver oil. The storage in the livers of newborn calves from cows receiving abundant vitamin A was found to be relatively low. Attention is called to the low concentration of vitamin A in the livers of calves at birth and to its high concentration in colostrum milk. This parallels the low concentration of globulins in the serum of newborn calves and their high concentration in the colostrum.

In these experiments the manifestations of vitamin A deficiency occurring under natural conditions on the range, which were reported in previous publications (Hart et al., '33 a, '33 b) have been produced under controlled conditions. The relation of these findings to problems in range cattle in California is discussed.

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VITAMIN A STORAGE IN THE LIVERS OF TURKEYS AND CHICKENS

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ONE FIGURE

(Received for publication October 23, 1933)

Under conditions commonly prevailing in commercial poultry production in California, supplying vitamin A is an important problem. Since fresh green feed is frequently limited, definite quantities of alfalfa meal or leaf meal are fed primarily to furnish this dietary essential. Nevertheless, symptoms of vitamin A deficiency sometimes occur in birds on rations that are generally considered adequate. Avitaminosis-A occurs also in range raised turkeys during the drought period when the food consists largely of seeds, dried herbage and insects. Under these conditions exact feeding history is often not available, and the manifestations are varied and indefinite or obscured by other complications.

The investigations reported in this paper on the application of the Carr-Price ('26) color reaction for estimating vitamin A storage in livers were undertaken with the hope of making this technic an aid to diagnosis, particularly in suspected hypovitaminosis where historical, clinical, and pathological findings are not decisive.

EXPERIMENTAL

Vitamin A storage in the livers of turkeys and chickens fed various levels of dehydrated alfalfa leaf meal

The birds used in this study were hatched and reared by the Division of Poultry Husbandry. They came from an investigation on the comparative pathology of avitaminosis-A

in chickens and turkeys, which will be reported elsewhere. As the present work was not outlined until near the close of the experiment, data regarding birds on low levels of vitamin A under 7 months of age were not available. The chickens were single comb White Leghorn females, while the turkeys were of the Bronze variety and about equally divided as to sex. The basal ration in the avitaminosis-A experiment was as follows:

	Pounds
Ground white corn,	25
Ground barley,	25
Ground wheat,	25
Fish meal,	10
Dried skim milk,	10
Bone meal,	3
Ground limestone,	2
Sodium chloride,	0.5

Vitamin A was supplied by adding freshly cut alfalfa or dehydrated alfalfa leaf meal containing approximately 130 gamma of carotene per gram in the proportions indicated in table 1. The mash was fed in hoppers to which the birds had access at all times. They were also exposed to direct sunlight for several hours daily.

The data on the liver storage of vitamin A presented in table 1 were obtained by quantitative estimation of the blue color produced by antimony trichloride reacting with chloroform solutions of the liver oils, a dilution procedure being used. The technic has been given in detail in another publication (Guilbert and Hart '34).

In each lot designated in table 1, the chickens and turkeys were kept in the same pens and fed from the same hoppers so that their feeding and environmental conditions were identical. The data show a direct correlation between the amount of dehydrated alfalfa leaf meal in the ration and the storage of vitamin A in the livers as represented by the color test. The chickens, though about 1 week younger than the turkeys when autopsied, showed greater storage of vitamin A than the turkeys on comparable rations.

Average growth curves of the various lots are presented in figure 1 which show that the growth of the turkeys was more retarded than that of the chickens fed comparable levels of dehydrated alfalfa leaf meal. The limitation of growth in the turkeys on the 2 per cent level (lot 4) was comparable to that of the chickens fed at the 1 per cent level (lot 5). There was heavy mortality among the turkeys and none among the chickens in these lots. All the turkeys on the 1 per cent level

TABLE 1

Vitamin A storage in the livers of chickens and turkeys fed various levels of vitamin A from hatching to autopsy at 28 to 30 weeks of age

LOT NO.	RATION	NUMBER OF LIVERS TESTED		AVERAVE NUMBER OF UNITS PER GRAM OF LIVER[1]	
		Chickens	Turkeys	Chickens	Turkeys
1	Basal ration + fresh alfalfa 20 weeks; 8 per cent D.A.M.[2] last 8 weeks	1	2	250	100
2	Basal ration + 8 per cent D.A.M.	4	3	270	65
3	Basal ration + 4 per cent D.A.M.	3	3	150	30
4	Basal ration + 2 per cent D.A.M.	4	4	36	1+
5	Basal ration + 1 per cent D.A.M.	4	..	4	...

[1] In our work a unit is defined as the amount of chromogen in 0.05 to 0.2 cc. of test solution which will give with 2 cc. of $SbCl_3$ reagent the faintest detectable blue color, using the technic outlined by Guilbert and Hart ('34). This amount of blue color is equivalent to that produced by 1 to 1.5 gamma of crystalline carotene (S. M. A. Corporation. M.P. 166° to 168°). The units reported here should not, therefore, be confused with blue units based upon the Lovebond tintometer.

[2] D.A.M. = dehydrated alfalfa leaf meal.

of leaf meal died before reaching 20 weeks of age, and symptoms of avitaminosis-A occurred among the turkeys even on the 4 per cent level. None of the chickens, on the other hand, manifested clinical evidence of vitamin A deficiency except for mild indications, toward the end of the experiment, in some of those on the 1 per cent level. Thus the growth and clinical data confirm the results of the color test.

The experiment was repeated with four additional lots of later hatched turkeys, omitting the 1 per cent level of de-

hydrated leaf meal. These birds did not do so well on the 2 per cent and 4 per cent levels of leaf meal as those in the

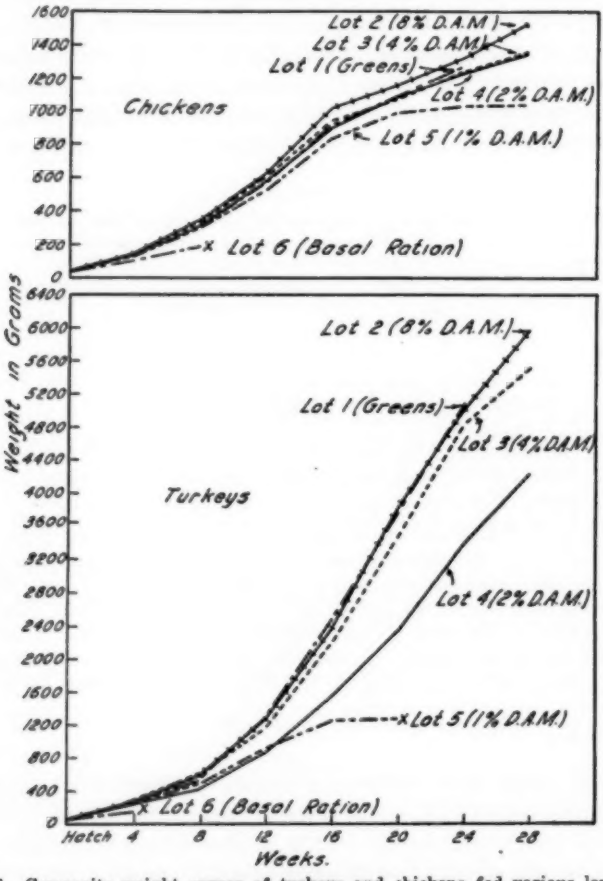

Fig. 1 Composite weight curves of turkeys and chickens fed various levels of vitamin A. (D.A.M. = dehydrated alfalfa leaf meal.)

first experiment, and the mortality was higher. Although the storage in the livers followed the trend shown in table 1 it was consistently lower.

Correlation between vitamin A storage in the liver as determined by the color test and period of survival of birds from the same lot when placed on the basal ration. At the time when the birds were autopsied to secure the data presented in table 1, three additional turkeys showing no visible evidence of deficiency were selected from each lot and placed on the basal ration. Three chickens from lot 4, and three from lot 5 were similarly treated. The birds in lots 3, 4 and 5 that were

TABLE 2

Correlation between vitamin A storage in the livers and the survival period of penmates placed on the basal ration

LOT NO.	ORIGINAL RATION	UNITS PER GRAM OF LIVER AT 30 WEEKS	TIME AFTER PLACING ON BASAL RATION		
			First clinical symptom (days)	First death (days)	Last death (days)
1 (turkeys)	Basal ration + green alfalfa	100	96	...[1]	140
2 (turkeys)	Basal ration + 8 per cent D.A.M.[4]	65	87	107[2]	135
3 (turkeys)	Basal ration + 4 per cent D.A.M.	30	56	90	...[3]
4 (turkeys)	Basal ration + 2 per cent D.A.M.	1+	33	53	86
4 (chickens)	Basal ration + 2 per cent D.A.M.	36	56	60	85
5 (chickens)	Basal ration + 1 per cent D.A.M.	4	14	24	37

[1] Two birds killed to determine the blue value at the time of first clinical symptoms.

[2] One bird killed ninety-sixth day which probably would not have lived to the one hundred and seventh day.

[3] Remaining birds at 90 days were in advanced stages of deficiency and were autopsied.

[4] D.A.M. = dehydrated alfalfa leaf meal.

placed on the basal ration were in better condition than those autopsied at 30 weeks. Since the birds autopsied represented as nearly as possible an average, including the better birds as well as those in poor physical condition, their storage is not strictly comparable to that of the birds placed on the basal diet. The data are presented in table 2.

Table 2 shows a direct correlation between the blue values found in the livers of birds autopsied at 30 weeks and the survival period of penmates placed on the vitamin A deficient basal ration.

Birds autopsied upon showing the first clinical symptoms of vitamin A deficiency gave blue values for the liver extract varying from a trace to 2 units per gram. Birds in this condition usually survived 20 to 30 days. In lot 4, therefore, the turkeys placed on the basal ration evidently had greater storage than their penmates autopsied at 30 weeks of age. No blue color was obtained with antimony trichloride reagent from any of the liver extracts of numerous cases, both chickens and turkeys, that were killed in cachexia or died of vitamin A deficiency.

TABLE 3

Comparison of vitamin A storage in the livers of normal turkeys of various ages

AGE	NUMBER OF LIVERS TESTED	AVERAGE UNITS PER GRAM OF LIVERS	EXTREMES: UNITS PER GRAM OF LIVERS	REMARKS
10 weeks	6	50	.....	Average of 2 composite samples from 3 males and 3 females
17 weeks	4	335	140– 400	Average of 4 individual determinations; 2 males and 2 females
21 weeks	4	620	500– 800	Average of 4 individual determinations; 2 males and 2 females
12–15 months	19	970	300–2000	Average of 8 individual and 3 composite samples. All females
2 years or older	11	2000	1300–3000	Average of 3 composite and 2 individual samples. All females

Vitamin A storage in the livers of turkeys fed high levels of vitamin A

The turkeys used for this phase of the studies were raised, from hatching to time of autopsy, on rations abundantly supplied with vitamin A. The mash contained 17 to 25 per cent yellow corn and 5 per cent dehydrated alfalfa leaf meal. In addition, the birds had access to all the fresh greens they would consume. For the first 4 weeks after hatching, fresh green lettuce was fed; from then on freshly cut and chopped alfalfa.

The variation of vitamin A storage in turkeys of different ages on this ration is shown in table 3.

The 10, 17 and 21 weeks old groups were hatched in 1933 from closely related parent stock. The 12 to 15 months old groups had completed the first laying period; the older birds, two or more laying seasons. As the data show, liver storage of vitamin A gradually increases during growth, reaching the highest values at maturity. This finding agrees with numerous data on other species.

Considerable variation existed in the liver storage of individuals within an age group. No correlation to explain it was found between liver storage and egg production or the functional state of the ovaries at the time of autopsy nor were sex differences noted.

Chickens of comparable ages were not available for comparison with the turkeys. Ten individual determinations were, however, made on the livers of 8 months old females fed essentially the same ration as the growing turkeys referred to in table 3. The average value found was 1150 units per gram. Two composite samples, each consisting of three livers from penmates, were taken at 12 months of age. These gave values of 4000 and 5000 units per gram, respectively. At 8 months the birds were practically mature and were producing eggs. Apparently the ration contained sufficient vitamin A not only for heavy production but also for significant storage, as judged by the values obtained for the penmates at 12 months of age.

The chickens at 8 months of age may be considered comparable in maturity to turkeys 12 to 15 months old. The liver storage at these ages again shows the tendency toward higher values in the chickens than in the turkeys fed similar rations.

Individual variation in storage of vitamin A

Attention has already been called to individual variation in the vitamin A liver storage of turkeys. Of the ten individual liver samples from 8 months' old chickens mentioned in the preceding paragraphs, four gave values varying from 1000 to 2500 units per gram; three gave values of 500 to 700

units; while two gave values of 100 units each and the remaining sample contained only 20 units per gram. This last value, at first thought to result from faulty technic, was checked by a second set of determinations on this liver. These birds were from the same source and from closely related stock, had received the same feeds, and were all producing eggs at the time of autopsy. (Trapnest records were not available.)

The variation in the liver storage of the normally fed birds, both chickens and turkeys, which had free access to green feed appears much greater than in the experimental lots where all the vitamin A carrying feeds were incorporated in the ration in a manner that made selection difficult. The results suggest that the variation in storage may be partly caused by individual differences in food habits. The variations found by the color test are comparable to those commonly noted in biological tests. Sherwood and Fraps ('32), for example, reported a variation of 35 to 199 days in the life of White Leghorn pullets after changing from an adequate to a vitamin A free diet. The average survival was 135 days.

A large error in expressing liver storage on the basis of a unit weight of liver tissue lies in the variation of the weight of the organ depending on the storage of glycogen and other products of digestion. The liver weights tend to become constant after 24 hours of fasting. The average weight of livers from twelve turkeys fasted 24 to 48 hours was 67 gm.; from seven non-fasted birds, 99 gm. The difference in fasted and non-fasted chickens was usually greater than in the turkeys. For example, the average liver weight of three chickens fasted 48 hours was 18 gm., as compared with 37 gm. for a like number of non-fasted birds. The livers of non-fasted chickens were commonly much lighter in color and more friable than those of fasted birds. The significance of the weight variation was not fully appreciated during the early part of this investigation. Fortunately, however, practically all our data are based upon liver samples from birds which had been fasted 24 hours or more before autopsy.

Observations on the nature of the chromogenic substances in livers

Chickens of the yellow-skinned breeds have more highly pigmented fat and liver extracts than turkeys of comparable maturity receiving the same feeds. One may therefore logically inquire whether the higher values found for chickens are partly caused by these pigments rather than by vitamin A.

Palmer ('22) has shown that the pigment of the body fat of chickens is largely xanthophyll and we have found this to

TABLE 4

Distribution of total chromogenic value with antimony trichloride between the petroleum ether and the alcohol fractions of liver extracts

SAMPLE	SPECIES	TREATMENT BEFORE AUTOPSY	TOTAL UNITS PER GRAM OF LIVER (DIRECT DETERMINATION)	PETROLEUM ETHER FRACTION—UNITS PER GRAM OF LIVER	85 PER CENT METHYL ALCOHOL FRACTION—UNITS PER GRAM OF LIVER
Composite of 3 livers	Chicken	Fasted 48 hours	5000	300	5000
Composite of 3 livers	Chicken	Not fasted	2000	400	1700
Composite of 4 livers	Turkey	Fasted 24 hours	600	50	500
No. 88	Sheep	Carotenoid—deficient ration	1000	200	800
No. 28	Sheep	Carotenoid—deficient ration	....	30	1000

be true also in turkeys. The pigments of several liver extracts have been separated by the phase method. The non-saponifiable matter was taken up in a small quantity of low-boiling, light petroleum and this was extracted by means of four to six treatments with 85 per cent methyl alcohol. The distribution of the total chromogenic value between the petroleum ether fraction containing the carotene and the alcohol fraction containing the xanthophyll is shown in table 4.

The non-saponifiable matter from each fraction obtained from the first sample shown in table 4 was dissolved in 10 cc.

of chloroform. Comparison of the depth of yellow color in a colorimeter gave nearly equal amounts in the carotene and the xanthophyll fractions, whereas most of the chromogenic value with antimony trichloride was found accompanying the xanthophyll.

The liver weights of the non-fasted chickens (referred to in table 4) were twice those of the fasted birds. If allowance is made for the dilution caused by storage of glycogen, etc., the blue values would become about 800 units and 3500 units per gram of liver for the petroleum ether and alcohol fractions, respectively. Although the carotene is significantly reduced, the conversion to vitamin A appears far from complete after 48 hours of fasting.

In some samples most of the pigment in the non-saponifiable matter of turkey livers was extracted by alcohol—a fact showing that the yellow color was largely due to xanthophyll. In others, however, there appeared to be about equal amounts of pigment in each fraction. Since strong blue color reactions develop in the alcohol fractions from the chicken livers at dilutions where only faint yellow color is discernible, and since xanthophyll gives less color with antimony trichloride than does carotene (Gillam, Heilbron, Morton, Bishop and Drummond, '33) it is evident that most of the blue color in the alcohol fraction is produced by vitamin A. This laboratory is not equipped to make spectroscopic determinations. In the Plant Physiology Laboratory at Berkeley the alcohol fraction of one sample gave with antimony tricholoride a blue color having a very intense absorption band in the region of 610 mμ, while no perceptible band was observed in this region in the petroleum ether fraction. Several workers have, however, found vitamin A in both fractions; and the results obtained with the sheep livers shown in table 4 and with cod liver oil confirm these findings, as the non-saponifiable matter from these sources was practically colorless. A relatively large proportion of the total blue value obtained from chicken and turkey liver extracts may therefore be considered to come from vitamin A, particularly when the birds have been fasted.

SUMMARY

A study of the Carr-Price reaction as a means for estimating liver storage of vitamin A in turkeys and chickens is reported.

A direct correlation was found between the liver storage (as expressed by the color test with antimony trichloride) and the level of vitamin A in the ration, the growth and mortality records, and the survival period of penmates when placed on the vitamin A deficient basal ration.

White Leghorn chickens were found to have greater storage of vitamin A than Bronze turkeys comparable as regards maturity and feeding history.

Large variations in liver storage were found among individuals from pens receiving the same feeds. It is suggested then, when selection is possible, individual variation in food habits may partly account for the variation in storage. Attention is called to similar variations in storage found in biological tests.

Turkeys receiving 8 per cent dehydrated alfalfa leaf meal showed a much lower liver storage of vitamin A than did turkeys having access to green feed in addition to vitamin A carrying feeds in their mash. Though the 8 per cent level of leaf meal sufficed for normal growth, the reserves were inadequate for protection against long periods of low vitamin A intake such as may occur on certain turkey ranges.

Data are presented on the correlation between vitamin A storage and age. Like other species, young growing turkeys had relatively little storage even on rations abundantly supplied with the vitamin. On such rations liver storage increased rapidly as percentage rate of growth decreased.

The blue value of liver samples taken at the time of the first clinical evidence of deficiency varied from a trace to 2 units per gram of liver. Birds in this condition survived from 20 to 30 days. Numerous tests on liver extracts from birds that died of avitaminosis-A or were killed in cachexia gave no blue color with antimony trichloride.

Though the intensity of the blue color reaction using the technic described by Guilbert and Hart ('34) has limitations from the standpoint of a precise quantitative method, the simplicity of the procedure and equipment renders it adaptable to general use. As shown by the data here presented, it may be a valuable aid to diagnosis and may also add materially to the information obtained by experiments on vitamin A requirements of fowls.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to W. E. Lloyd, Division of Poultry Husbandry, for his cooperation in furnishing the birds for these experiments and to R. W. Caldwell and H. C. Johnson for their assistance in the laboratory.

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VARIATIONS IN THE POTENCY OF CERTAIN FOODSTUFFS IN THE CURE OF DERMATITIS INDUCED IN RATS BY DIETARY EGG WHITE [1]

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(Received for publication November 9, 1933)

It has been found that dietary egg white is capable of producing severe dermatitis and nutritive failure in both rats and chickens when it is incorporated in certain well supplemented rations,[2] even when the vitamin G content is in excess of the amount sufficient to insure growth and protection from dermatitis in ordinary diets. When the egg white is treated in one of various ways, or the concentration of certain potent foodstuffs in the diet is sufficiently high, however, dermatitis may be cured and nutritional well-being restored.[2]

Although the syndrome produced by means of these presumably adequate rations containing egg white is strikingly similar to that occurring on purified diets low in vitamin G, doubt that the protective factors involved in the two cases are identical has been occasioned by finding certain differences in distribution and resistance to extraction by solvents (Boas, '27; Parsons and Kelly, '33). The present experiments were undertaken to investigate the nature of the factor

[1] This work was aided in part by a grant from the University of Wisconsin Research Fund. A preliminary report of a part of this investigation was presented to the American Society of Biological Chemists at Cincinnati, April 11, 1933 (Parsons, H. T., J. G. Lease and E. Kelly, 1933, J. Biol. Chem., vol. 100, p. lxxvii; Proc. Am. Soc. Biol. Chem., 1933, vol. 8, p. lxxvii). Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

[2] The literature has been reviewed by Parsons ('31) and by Parsons and Kelly ('33).

or factors protective against dermatitis due to egg white. In this paper, only the general sources and properties of the factor as revealed by manipulations aimed at its isolation are reported.

EXPERIMENTAL

The characteristic syndrome due to dietary egg white was produced in albino and hooded rats on ration B as previously described (Parsons and Kelly, '33). When soreness of the lip was definitely established on this ration, a given foodstuff or preparation to be tested was fed in basal ration S, replacing part of the sucrose. This ration contained 40 per cent of Chinese dried egg white, 10 of wheat embryo, 4 of Osborne and Mendel ('19) salt mixture and 46 of sucrose with daily additions of 3 to 5 drops of cod liver oil. When the preparation to be tested lacked the vitamin B complex, 10 per cent of yeast was also substituted for an equivalent weight of sucrose in the basal ration (ration H). Without the addition of potent substances other than the amount of yeast and wheat embryo used, both rations B and H lead to decline and frequently to death within the 30-day test period following the establishment of the symptoms. The potency of the preparations tested was judged by the same criteria that have been used previously in determining the loss of toxicity in heated egg white (Parsons and Kelly, '33).

Unless otherwise stated, the various foodstuffs to be tested were fed after thorough cooking and drying.

Hemoglobin determinations were made by the Newcomer method with the use of a calibrated colored glass disk.[3]

RESULTS

Occurrence of the potent factor. No systematic investigation of foodstuffs as sources of the factor was attempted; the materials tested were those which gave promise of being rich in the factor or were otherwise especially suitable for investigation.

[3] We are indebted to Dr. C. A. Elvehjem, of the Department of Agricultural Chemistry of the University of Wisconsin, for calibrating our calorimeter.

Cooked pork liver and beef kidney proved to be about as effective as the cooked beef liver previously reported (Parsons, '31). Cooked pork kidney was one and one-half times as potent as beef liver, and ranks as the richest source of the factor protective against the dermatitis due to egg white which has thus far been discovered; a 5 per cent[4] concentration in basal ration S led to pronounced improvement in the dermatitis (table 1). Negative results with ½ of 1 per cent of adrenal glands (beef) indicated that the potency of the kidney tissue was not attributable to a possible contamination with adrenal tissue. Seven per cent of beef ovary, 7 and 30 per cent of beef heart, 5 per cent of pork heart, 7 and 30 per cent of beef spleen, and 5 per cent of pork spleen were entirely ineffective in improving the condition of the rats. Hemoglobin fed at 3 per cent, cooked beef blood at 7, and raw dried dog blood at 10 per cent were entirely ineffective in relieving the dermatitis due to egg white, in spite of the fact that they furnished much higher concentrations of hemoglobin than were present in the curative doses of liver or kidney used in our experiments. These results are not in harmony with the published statement of Bliss and Thomason ('30–'31) that dried whole dog blood, dried hemoglobin and crystalline hemin prepared from dog blood were effective in curing the pellagra-like manifestations which these authors produced in rats on a non-purified diet of natural foodstuffs in which raw egg white furnished the chief source of protein.

Five times the percentage of ferric citrate found in Osborne and Mendel's salt mixture, and 0.5 mg. per day of copper fed as copper sulfate, or these fed together were entirely ineffective for either protection or cure as was the ash of 20 per cent of beef liver. Furthermore, a study of the hemoglobin concentration[5] in the blood of the rats on egg white

[4] Percentages of this and other preparations are expressed on the air-dry basis.

[5] Credit is due to the following senior students in the Department of Home Economics for their assistance in making simultaneous hemoglobin determinations with one of the authors in many of the experiments: Gertrude Irwin, Catherine Johnson, Jeanette Lepp, Milada Prochaska and Norma Vesperman.

diets showed that although the average values tended to be somewhat lower than the average for rats on very good diets, they fell within the range considered normal for rats from 50 to 90 days of age (Williamson and Ets, '26) and did not

TABLE 1

Comparative potency of certain foodstuffs and preparations in curing the dermatitis due to egg white

FOODSTUFFS [1]	CONCENTRATION OF SUPPLEMENT WHICH IS				CONCENTRATION OF EGG WHITE IN RATION
	Very potent	Moderately potent	Slightly potent	Not potent	
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Raw beef liver				7, 10, 15	40
Raw pork liver			15	5	40
Raw beef kidney				5[3], 15[3]	40
Raw pork kidney		10	7		40
Cooked fresh beef liver	10	7	5	3	40
Cooked fresh beef kidney			5[3]	3	40
Cooked dried beef kidney		15[3]			40
Cooked fresh pork liver			5		40
Cooked dried pork liver	15				40
Cooked fresh pork kidney	7	5		1, 3[3]	40
Eli Lilly & Co. liver extract no. 343				10, 30	40
Liver residue from Eli Lilly & Co. extract		7			40
Wheat embryo	76				20
Wheat embryo		66			30
Yeast	50	40	30		40
Dried skim milk				60	40
Dried skim milk	80				20
Dried whole milk				50, 60	40
Dried whole milk			70		30
Egg yolk			60	40	40
Egg yolk		70			30
Raw beef liver, 37° 20 hours, then cooked	7		3		40
Raw beef liver, 23° 20 hours, then cooked	7				40
Raw beef liver, + N/50 HCl 37° 10 days		7			40
Raw beef liver, + N/50 HCl 37° 10 days then cooked	7				40
Raw beef liver, 25° 22 days then cooked		7			40

TABLE 1—*Continued*

FOODSTUFFS [1]	CONCENTRATION OF SUPPLEMENT WHICH IS				CONCENTRATION OF EGG WHITE IN RATION
	Very potent	Moderately potent	Slightly potent	Not potent	
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Cooked pig kidney, moist 25°, 16 days		3	1		40
Raw fresh beef liver, 6 days at 100°, kept moist				7, 15	40
Raw fresh beef liver, 6 days at 100°, dry				7	40
Raw dried beef liver 3½ days at 100°, dry			7		40
Raw dried beef liver 6 days at 100°, dry		15			40
Cooked beef liver, dried, 3½ days at 100°, kept moist				7	40
Cooked beef liver, dried, 3½ days at 100°, dry				7	40
Cooked beef liver, dried, 6 days at 100°, kept moist				15	40
Cooked beef liver, dried, 6 days at 100°, dry				15	40
Raw pork kidney, dried, 3½ days at 100°, kept moist		7			40
Raw pork kidney, dried, 3½ days at 100°, dry			7		40
Cooked pork kidney, dried, 3½ days at 100°, dry	7				40
Raw fresh pork kidney, 6 days at 100°, dry		7			40
Raw pork kidney, dried, 6 days at 100°, dry			7		40
Cooked pork kidney, 6 days at 100°, kept moist	7				40
Cooked pork kidney, 6 days at 100°, dry				7	40

[1] Foodstuffs were fed dry; the weight given is on the air-dried basis.

[2] These groups had equalized food consumption.

decrease with the length of time on the egg white rations. For example, a group of nineteen rats showed an average hemoglobin concentration of 13.3 gm. per 100 cc. of blood (ranging from 10.6 gm. to 15.5 gm.) after 35 to 45 days on the

egg white diet, the period just preliminary to the appearance of sore lips and other manifestations of dermatitis. Also no correlation was found between the hemoglobin concentrations at weaning when the egg white diet was begun and the rate at which the symptoms appeared.

Since it seemed possible that a dehydration of the blood might be masking an actual anemia, blood solids were also determined in one series. Hardening and necrosis of the caudal tissues at the onset of dermatitis often interfered with taking blood samples from the tail, so that jugular blood samples were used in cases where the rats were sacrificed. The results showed that a group of nine rats, not exhibiting dermatitis, either because of protective foods fed or the short time on the ration, had an average concentration of 13.3 gm. of hemoglobin per 100 cc. of blood and 20.0 per cent of blood solids; thirteen rats with severe dermatitis, some in a critical condition, had an average concentration of 14.3 gm. of hemoglobin and 20.4 per cent of blood solids; and twelve rats, which had thoroughly recovered from a severe condition, had an average concentration of 12.9 gm. of hemoglobin and 20.6 per cent of blood solids.

In a few animals in which it was possible to obtain blood samples from the tail during an early period of recovery it was noted that hemoglobin values dropped markedly during the period of rapid gain in weight. For illustration, a rat in which severe dermatitis had been produced on ration B, had a blood hemoglobin concentration of 16.2 gm. per 100 cc. of blood. On changing the rat to a ration in which a favorable mixture of fresh egg white and fresh egg yolk was substituted for the Chinese dried egg white of the diet, a rapid cure ensued, with a gain in body weight of 6 gm. daily for 15 days and a drop in blood hemoglobin to a concentration of 12.4 gm. per 100 cc. during this time.

The failure of Eli Lilly & Co.[6] liver extract no. 343 to lead to a cure of dermatitis was striking. Fed as either 10 or 30 per cent of ration S, this concentrate exhibited no potency

[6] Kindly furnished by Eli Lilly & Co., Indianapolis.

whatever; the four rats on the dosage died before the expiration of the 30-day test period, with exaggerated symptoms of the disorder. These results are the more significant because of the high content of vitamin G in this liver extract. On the other hand, the solid liver residue[6] from the manufacture of the commercial liver extract was as potent in curing the dermatitis as cooked dried beef liver prepared in the laboratory (table 1). In accord with these results, certain other vitamin G-rich substances were relatively ineffective in curing the dermatitis due to egg white. It was necessary to include dried brewer's yeast[7] in at least as great a concentration as the egg white of the ration (i.e., 40 per cent) before distinct improvement was obtained. Wheat embryo, egg yolk[8] or dried milk could not be fed high enough with 40 per cent of egg white to lead to satisfactory cure. Only when the dried egg white of the ration was decreased to a concentration lower than 40 per cent, so that more of the supplementary food could be included and less toxicity was furnished by the egg white, could full recovery be secured (table 1). Freshly gathered mushrooms (Coprinus atramentarius) and watercress were cooked, dried and fed at a 10 per cent concentration (approximately the equivalent weight of the rest of the ration if they had been fed moist) without leading to any improvement in the condition of the rats.

Inasmuch as the most potent foodstuffs were rich in nucleoprotein, their effectiveness might possibly be attributable to this constituent. However, a sample of nucleoprotein prepared from beef liver and fed as high as 3 per cent—a concentration greater than that in which it occurred in rations

[7] From the Northwestern Yeast Company, Chicago.

[8] The results on egg yolk were confirmed and extended in a Bachelor of Science thesis by Arlette Caldwell ('31) written under the direction of Miss Dorothy Hussemann in the Department of Home Economics (unpublished data). In this study, dried egg yolk fed with dried egg white prepared from fresh eggs in the proportion of one-fourth egg yolk to one egg white did not prevent the onset of dermatitis when fed to weanling rats; but in the proportions found in whole egg (64 per cent of yolk to 36 per cent of white, dry basis) or as whole egg itself, it gave considerable protection as only doubtful or minor injury occurred during a 70-day feeding period.

containing potent supplements of liver[9]—failed to cause any improvement in the dermatitis. The residue from the nucleoprotein preparation, however, was approximately as potent as other cooked liver samples tested.

The possibility of loss of potency in liver tissue by various means was investigated. Beef liver and pork liver obtained immediately after the animals were slaughtered were of approximately the same potency as similar tissue procured from a packing house. Similarly, no loss of potency occurred in the fresh beef liver through such processes as the following: allowing the raw liver to stand for 10 days at 37° after the addition of N/50 HCl and chloroform to insure suitable conditions for autolysis according to the method of Herron and McEllroy ('32); or, without these additions, for 20 hours at 37°, or for 20 hours at room temperature, during which intervals extensive bacterial action took place. (See table 1 for full series.) The dried cooked product underwent only slow loss of potency through standing for long periods of time at room temperature, inasmuch as one such lot tested was found to have lost not more than one-third of its potency after a year's time.

It was conceivable that the apparent toxicity of egg white might in reality be a destructive action on the potency of the protective factor or factors in the mixed ration. Therefore, beef liver was tested which had been standing in the laboratory for 3 years incorporated in an egg white ration. It was fed at a 15 per cent concentration in ration S, to make allowance for the expected loss of activity at room temperature noted previously when dried liver was stored alone. The rapid recovery of the rats on this dosage gave evidence that no significant increase in the rate of destruction of the factor had been occasioned by contact with the egg white.

The potency of raw liver or kidney was distinctly increased by the heating of these products at water bath temperatures

[9] This was estimated from the 0.55 per cent yield of nucleoprotein obtained in this experiment, which was in good agreement with Wohlgemuth's ('02–'03) figures, i.e., 0.3 to 0.4 per cent of nucleoprotein from beef liver.

for 15 minutes or longer (table 1). Experiments with equalized food intakes confirmed this observation amply although the concentration of raw liver sufficient to furnish an adequate supply of the factor was not reached, because the food intake fell off as the concentration of raw liver or kidney was increased. From the data obtained, the potency appeared to be doubled or trebled by cooking. Increase in the activity was about equally well accomplished by cooking the fresh tissue or by drying and re-moistening it before cooking; but it was less satisfactorily accomplished by heating it dry at 100°, possibly because a certain amount of destruction accompanied the process.

Prolonged heating of liver, whether moist or dry, raw or previously cooked, resulted in pronounced destruction of the potent factor. In general, the same results were obtained with pork kidney. The data are presented in table 1. Heat appeared to be less destructive to the factor if the material were kept moist; other manipulations such as previous cooking or drying had an influence on the rate of destruction.

The process of boiling cooked beef liver with 10 per cent hydrochloric acid for 6 hours, with subsequent neutralization and drying, yielded a product in which no activity was demonstrated. Decreasing the time of boiling to 1 hour allowed the persistence of some activity; 1 hour's boiling with 5 per cent hydrochloric acid was only moderately destructive, about one-half to two-thirds of the potency being retained. The same extent of destruction resulted from a similar heat treatment of pork kidney with tenth normal sodium hydroxide. Treatment with this concentration of alkali in the cold for 4 days did not decrease the potency.

DISCUSSION

The results of these experiments offer conclusive evidence that the injury due to egg white is not in the nature of an amino acid deficiency, inasmuch as 30 per cent of dried beef heart or 60 per cent of dried milk in diets containing raw egg white, failed to effect a cure of the dermatitis. The pos-

sibility that an antienzyme might be involved is apparently also precluded. One possible hypothesis for an interrelationship of factors in a given diet, which has accounted for the damaging effects of a high content of lard and of iron compounds in certain rations, i.e., the destructive action of one ingredient upon another in a ration mixture, does not explain the present phenomena. If an immunological relationship shall be proved to be involved in the injury due to egg white in these experiments, it must function in a manner much more intricate than any known at present. The protection afforded by certain foodstuffs and the loss of this through certain agents, as demonstrated by these experiments suggests the action of a factor in the nature of a vitamin with the distinction that its action as investigated so far, appears to be to counteract a positive injury from egg white. The possible relationship of such a factor to the vitamin B complex will be discussed in a forthcoming paper.

SUMMARY

Cooked pork kidney is the richest source investigated, of the factor or factors which cure or prevent the dermatitis due to egg white, as it is necessary to include only one-sixth of the weight of the egg white present in a ration, for a cure. Cooked beef liver, pork liver and beef kidney are good sources, being effective if present in the ration in a concentration about one-fourth that of the egg white. One to three times the weight of the egg white, of dried yeast, dried egg yolk, wheat embryo or dried milk must be incorporated to be curative. There is relatively little or no potency in spleen, heart, ovary, adrenal, blood or hemoglobin. The activity resides in the solid liver residue left from the preparation of Eli Lilly & Co. liver extract no. 343, not in the extract itself. The potency of liver does not depend on its nucleoprotein fraction.

Cooking increases the activity of raw liver or kidney; autolysis or extensive bacterial action does not decrease it. Prolonged heating, i.e., 6 days at 100°C. is destructive, especially if the material is dry. Boiling with hydrochloric acid

for 1 hour at 5 or more per cent decreases the potency appreciably.

A low hemoglobin concentration is not a feature of the syndrome due to dietary egg white.

The injurious action of egg white apparently does not depend on the destruction of some dietary factor within the ration mixture.

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THE RELATION OF AVITAMINOSIS C TO BLOOD CLOTTING [1]

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(Received for publication October 16, 1933)

Several well-established observations upon blood changes which are due to avitaminosis C and which might be correlated with blood clotting are those of Morikawa ('20), Ohata ('30, '32), Randoin and Micheaux ('32), Micheaux ('31), Mettier and Chew ('32), Gronchi ('30), and Diblicek and Kucera ('33).

Bearing more directly upon the problem are the following investigations. Stockman ('03) has recorded, that in scurvy the blood coagulates normally. MacRae ('08) agrees with him but quotes Sir A. E. Wright who says that scurvy "eventuates in a defect in blood coagulability." These conclusions were founded upon observations of human subjects. Working with guinea pigs, Findlay ('21) found that scurvy had no effect upon blood clotting. Kugelmass ('32) states that deficiency in the clotting function in hemophilic children appears not to be altered by the vitamin content of the diet, but does not say that vitamin deficiency might not affect blood clotting. Ohata ('32) has found that in scorbutic guinea pigs not only is the blood coagulation time prolonged, but that there is a diminution in the number of blood platelets and in the amount of fibrinogen, thrombin and cephalin present.

[1] From a dissertation presented to the faculty of the Graduate School of the University of Cincinnati in partial fulfillment of the requirements for the degree of Master of Science.

[2] Merrell Fellow in Biochemistry.

It is quite possible that, in the older literature at least, there may have been some confusion of the avitaminoses A and C. For this reason, the present study of some of the blood changes, and particularly of the clotting time of the blood, was made upon animals whose diet contained all the essential factors with the sole exception of vitamin C.

EXPERIMENTAL

A. Diet. Thirty-four healthy guinea pigs, ranging in weight from 156 to 480 gm. were fed a basal diet of hay, oats, soy bean flour and sodium chloride, the latter three substances in the proportion of 65:35:1. In addition to this, eight of these animals received 4 cc. of fresh, unheated orange juice per day. The twenty-six experimental animals were given the same amount of orange juice which had been heated to destroy the vitamin C.

The animals fed the vitamin C free diet developed typical scurvy. Three of these assumed unquestionable 'face-ache positions.' Two of the scorbutic animals were completely cured by fresh orange juice after 37 days of the scorbutic diet. The controls remained healthy and grew normally.

B. Methods. Coagulation time was measured by means of Biffi-Brook's coagulometer, the blood being taken from the ear-vein. When this was impossible in certain severe cases of scurvy the blood was taken by heart puncture and a correction factor (0.9) applied.

The first series of platelets counts was made by the method of Fonio ('12), the rest by the method of Rees and Ecker ('23).

The red blood cells were counted with a Thoma Zeiss hemocytometer.

Hemoglobin was estimated by means of the Dare hemoglobinometer.

RESULTS

The lengthening of the blood coagulation time was the first apparent symptom of disease in twenty-one of twenty-six

cases. The prolongation of the clotting time became quite marked in the period immediately preceding death. It varied from 10 to 220 per cent, and averaged 54 per cent. The albinos showed a longer initial clotting time than did the others (tables 1 and 2).

In the scorbutic guinea pigs a reduction of platelets was found which averaged 36 per cent and which was closely parallel to the reduction of red blood cells (tables 3, 4, 5 and 6).

TABLE 1

Clotting time (in minutes). Group of eight guinea pigs on a scorbutic diet plus 4 cc. orange juice per day

	3/2/33	3/8/33	3/14/33	3/20/33	3/26/33	4/1/33	4/7/33	4/13/33	4/19/33	4/25/33	PER CENT INCREASE
Maximum	3.50	3.25	3.50	3.50	3.50	3.50	3.25	3.50	3.25	3.25	+ 10
Minimum	2.50	2.50	2.50	2.50	2.75	2.50	2.25	2.25	2.25	2.25	— 18
Average	2.97	2.91	2.84	2.84	2.97	2.91	2.81	2.75	2.72	2.72	— 8

TABLE 2

Clotting time (in minutes). Group of twenty-four guinea pigs on scorbutic diet

	3/2/33	3/8/33	3/14/33	3/20/33	3/26/33	4/1/33	4/7/33	4/13/33	PER CENT INCREASE
Maximum	4.25	4.75	5.00	5.00	7.20	5.00	5.00	6.30	+ 220
Minimum	2.25	2.25	2.25	2.25	2.50	2.75	2.75	2.75	+ 10
Average	2.34	2.52	2.88	3.05	3.36	3.28	3.49	4.92	+ 54

A decrease in hemoglobin was detected in the blood of the scorbutic animals, but as the disease progressed the blood underwent a slight change in color which prohibited the continuation of this determination.

In attempted estimations of anticoagulant it was noticed that if blood from a scorbutic guinea pig is allowed to clot and is then centrifuged or given an opportunity to synerese the proportion of serum obtained is two to three times as great as that obtained from normal blood by the same process.

TABLE 3

Platelet counts. Group of eight guinea pigs on a scorbutic diet plus 4 cc. orange juice per day

	3/2/33	4/1/33	4/13/33	4/25/33	PER CENT DECREASE
Maximum	370,000	390,000	390,000	390,000	+ 11
Minimum	320,000	320,000	320,000	320,000	— 15
Average	345,000	360,000	355,000	353,000	— 2

TABLE 4

Platelet counts. Group of twenty-four guinea pigs on a scorbutic diet

	3/2/33	4/1/33	4/13/33	PER CENT DECREASE
Maximum	390,000	290,000	270,000	+ 54
Minimum	310,000	190,000	150,000	+ 22
Average	348,000	233,000	188,000	+ 36

TABLE 5

Red blood cells. Group of eight guinea pigs on scorbutic diet plus 4 cc. orange juice per day

	3/2/33	4/1/33	4/13/33	4/25/33	PER CENT DECREASE
Maximum	6,700,000	6,500,000	6,500,000	6,700,000	+ 11
Minimum	5,700,000	5,600,000	5,900,000	5,800,000	— 8
Average	6,100,000	6,200,000	6,100,000	6,100,000	— 1

TABLE 6

Group of twenty-four guinea pigs on scorbutic diet

	3/2/33	4/1/33	4/13/33	PER CENT DECREASE
Maximum	6,700,000	5,000,000	4,200,000	+ 47
Minimum	5,700,000	3,500,000	3,100,000	+ 14
Average	6,100,000	4,500,000	3,700,000	+ 28

CONCLUSIONS

It was found that the blood of scorbutic guinea pigs has a longer clotting time, a smaller number of blood platelets and of red blood cells, and a smaller amount of hemoglobin than does the blood of normal guinea pigs. It was also found that a greater proportion of the blood of scorbutic guinea pigs separates as serum than is the case in the blood of the healthy animals.

These changes in the blood begin before any other of the usual symptoms of scurvy appear, and it is possible that incipient scurvy in guinea pigs may thus be recognized from the blood changes before it is possible to detect it in other ways.

Whether in human beings these blood changes occur early in the disease has not been investigated.

These changes were found not to take place in young female albino rabbits in twice the time required for guinea pigs to die of scurvy when fed the same diet.

I wish to express my gratitude to Prof. A. P. Mathews for his helpful criticisms and suggestions.

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THE DEVELOPMENT OF XEROPHTHALMIA AND THE KERATINIZATION OF EPITHELIAL TISSUE ON WITHDRAWAL OF VITAMIN A FROM THE DIET OF THE MONKEY (MACACUS RHESUS), GUINEA PIG, RABBIT, AND ADULT ALBINO RAT [1] [2]

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SIX TEXT FIGURES AND FOUR PLATES (TWENTY-TWO FIGURES)

(Received for publication October 13, 1933)

In man (Wilson and DuBois, '23), in the albino rat (Wolbach and Howe, '25), and in the albino mouse (Wolfe and Salter, '31), a specific effect of vitamin A deficiency has been found in the development of an eye disease usually described as keratomalacia in man and xerophthalmia or ophthalmia in animals, and in a change in the epithelial structures resulting in the substitution of stratified keratinizing epithelium for normal epithelium in the respiratory tract, alimentary tract, genito-urinary tract, eyes, and para-ocular glands. It was observed by Daniels et al. ('23) that rats afflicted with xerophthalmia often showed suppurations in the nasal cavities and sinuses. When there has been a lack of vitamin A in the diet, xerophthalmia has been observed in the rabbit (Nelson and Lamb, '20), the chicken (Beach, '23), the dog (Steenbock et al., '21; Stimson and Headley, '33), and in cattle (Hart et al., '33). The cattle observed by these last-named authors showed not only xerophthalmia with severe corneal involve-

[1] Conducted under a grant from the Commonwealth Fund of New York.

[2] The observations reported in this paper were made on vitamin A depleted animals used in a study of the nutritional aspect of trachoma which will be reported in another paper.

ment but also lung involvement with difficult respiration, emaciation, and severe diarrhea. Analysis of liver samples of the cattle dead from vitamin A lack in the diet showed no vitamin A in the liver.

Clouding of the cornea but no typical conjunctivitis was observed by Boock and Trevan in the guinea pig ('22). Wolbach and Howe were unable to demonstrate the eye disease in guinea pigs but they did find extensive keratinization of the epithelial tissues of the uterus, the ducts of the submaxillary glands, the trachea, and the bladder ('28).

Xerophthalmia was reported by Saiki ('29) in two very small monkeys after 30 and 47 days, respectively, on a diet free from vitamin A (Saiki, '29). Turner and Loew ('31) fed the Saiki diet to monkeys but failed to produce xerophthalmia. Nor did their monkeys show any signs of keratinization of the epithelial tissues studied. The animals succumbed after 1 to 10 months on the diet with a loss of weight, loss of appetite, and gastro-intestinal symptoms. Postmortem examination showed evidence of intestinal inflammation, marked enteritis, and dilatation of the stomach. The upper respiratory tract, nasal cavities, and middle ear were free from suppuration in all of their deficient animals. Turner and Loew also fed the same vitamin A low diet used by McCarrison ('20) but failed, as McCarrison had done, in showing any specific results characteristic of the deficiency.

Tilden and Miller ('30) again failed to produce xerophthalmia in monkeys even after 312 days on a ration containing only six to twelve units of vitamin A daily. There was no sinusitus or mastoiditis but in nine out of eleven monkeys keratinization occurred in the epithelial tissue of the seminal vesicle, the ureter, the bladder, or at the gastroesophageal junction. The animals reported by Tilden and Miller lived from 80 to 312 days on the vitamin A-low diet. In all of the monkeys studied there was a loss of weight followed by a loss of appetite and finally by severe colitis and death.

It has been generally concluded from these recent observations of Tilden and Miller and Turner and Loew that vitamin

A depletion in the monkey does not lead to the eye disease, xerophthalmia, but rather to a gastro-intestinal pathology. Observations made in our laboratory, however, indicate that a monkey depleted of vitamin A may react in the same characteristic manner as does the rat deprived of this vitamin. While xerophthalmia developed in only one of the twenty-seven monkeys under observation, this animal was the only one that lived longer than the monkeys reported by previous investigators. This animal remained in fairly good health for almost a year without showing severe colitis or loss of appetite and after 13 months on the diet showed keratomalacia of both eyes. The specific effect of the vitamin A deficiency was manifested in the substitution of stratified keratinizing epithelium for normal epithelium in some of the epithelial structures. This specific effect in addition to the development of xerophthalmia was also observed in vitamin A depleted guinea pigs, rabbits, and rats under observation in this investigation.

EXPERIMENTS WITH MONKEYS

Young monkeys, Macacus rhesus, weighing from 1 to 2 kg. each, were housed in individual heavy metal mesh cages with wire bottoms of large enough mesh to allow feces to fall through. The drinking cups of 500-cc. capacity were of monel metal and built into the door of the cage. The removable pans below the wire bottoms were also of monel metal. The cages and pans were cleaned three times weekly and the pans were sometimes cleaned oftener. Sawdust or shavings were kept in the pans.

The monkeys received food which was very low in vitamin A. The diet included a combination of rolled oats, vitamin A-free casein, cornstarch, sugar, hydrogenated vegetable oil[3], egg white, Osborne-Mendel salt mixture, sodium chloride, and water mixed and baked into a cookie which was readily eaten by the monkeys. This mixture, called monkey 'cookie,' made according to the formula given in table 1, furnished most of

[3] Crisco.

the protein, carbohydrate, fat, minerals, and vitamins B, G, and E of the diet. The diet also included tomato serum,[4] a clear yellow juice which was shown by the writer ('30, unpublished data) and by Steenbock and Schrader ('31) to be almost lacking in vitamin A. It was also shown by the writer ('30, unpublished data) to be an excellent source of vitamin C. The tomato serum was used especially for its vitamin C contribution to the diet but it also added vitamins B and G, minerals, and carbohydrate to the daily food intake. From 3 to 4 ounces of tomato serum were given daily with the water. The

TABLE 1

Formula for monkey 'cookie'

	Gm.	*Per cent*
Casein (alcohol extracted)	75	5
Oats, rolled	675	45
Cane sugar	375	25
Sodium chloride	15	1
Salt mixture (Osborne-Mendel)	60	4
Cornstarch	150	10
Hydrogenated vegetable oil[1]	150	10
Egg white	500	
Water	500	

After the above dry components were mixed thoroughly the hydrogenated vegetable oil[1] was added and the whole mixture combined with the egg white and water. It was then spread about ¼-inch thick on flat baking pans and baked for 3 to 4 hours in a moderately hot oven, when it was cut into strips about 1 × 3 inches in size and baked for an hour or more longer to further dry the cookies.

[1] Crisco.

diet also included 6 drops daily of Viosterol, 250 D, for vitamin D. This was given in the cup with the tomato serum and water.

A weighed amount of monkey 'cookie' was given to each animal daily for the first 4 months of the feeding. Considerable of the food, however, was lost through the wire mesh bottom of the cage and therefore for the remainder of the experiment, monkey 'cookie' was given ad libitum. It was estimated that the animals ate daily of the 'cookie' and tomato serum enough food so that their average daily intake

[4] Tomato serum was purchased from the Welch Grape Juice Company.

was approximately 140 calories and 4.5 gm. of protein per kilogram body weight. The calories were distributed as shown in table 2.

TABLE 2

Average distribution of calories in the foods eaten daily by the monkeys (This includes the monkey 'cookie,' tomato serum, and Viosterol)

	Per cent
Protein	13
Carbohydrate	60
Fat	27

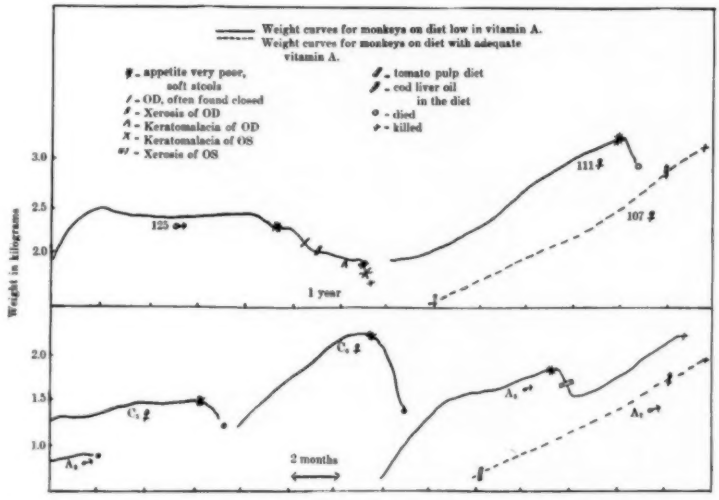

Chart A Weight curves of monkeys with and without vitamin A in the diet.

The monkeys were usually weighed every week and record made of the appetite and condition of the stools. If the appetite was below normal or the stools were soft, 5 to 10 gm. of dried yeast[5] were given daily for a few days or until the appetite and stools were again normal. The first effect of the yeast was to produce diarrhea which soon ceased but would always reappear if the yeast was continued for too long a period.

[5] Northwestern.

Some of the monkeys were in a very healthy condition when the feeding was begun but a few of them were very thin and unkempt in appearance when received. Of the 27 animals obtained for the study, 2 died from tuberculosis, 6 lived less than a month on the diet, 1 lived for 2 months, 4 lived for 3 months, 1 lived 4 months, 6 lived from 6 to 7 months, 1 lived from 9 to 10 months, and 1 monkey, 125, was killed after 13 months on the diet. Six of the depleted animals were fed tomato pulp for vitamin A after loss of appetite and loss of

Fig. 1 Monkey 125, showing xerophthalmia. The cornea of the right eye had perforated. The left eye is beginning to show keratomalacia.

weight occurred. Autopsy was made of all of the animals and histological examination was made of some of the tissues of monkeys 125, 111, S_4, and C_5. The livers of monkeys 125, 111, C_4, C_5, A_8, and of a normal control were fed to vitamin A depleted rats to test for the presence of vitamin A.

Chart A shows typical weight curves of monkeys fed the vitamin A-low diet. Of greatest interest is the record of monkey 125. He was one of the first two monkeys given the diet in October, 1931. Both monkeys gained weight rather rapidly for a time and for 8 months were very healthy in

appearance. The fur then began to look slightly ragged and the appetites were none too good. Yeast was given daily for a week and the appetites improved but in June one of the monkeys died and at autopsy tubercles were found. To try to save monkey 125 so that the lack of vitamin A in the diet could be studied, this animal's cage was moved for the summer to the roof in the sunshine, and the daily intake of tomato serum was doubled. His appetite remained good and the stools were fairly well formed until late in July when the appetite was poor again and there was mild diarrhea. When yeast was again given the appetite improved as also did the stools but the weight was decreasing. Tomato serum was offered ad libitum and sometimes as much as 16 ounces were ingested daily. During the last of August, 10 months after the feeding was started, the right eye of this monkey was often found closed and he avoided looking at the light. The teeth began to loosen and fall out, but before the monkey was killed new teeth came in to replace the lost ones. Before the twelfth month on the diet, the right eye showed definite xerosis, the cornea was wrinkled, and there were Bitot's spots. The left eye was at times found completely closed. A corneal ulcer soon developed on the right eye and perforated. During the thirteenth month on the diet, the left eye showed the same changes which had occurred in the right eye, and as soon as the cornea became opaque, the animal was killed. Figure 1 shows a picture of the monkey after 13 months on the diet. He was somewhat inactive, lean and emaciated, and the hairy coat was very rough. There was no discharge from the nose and no severe diarrhea, although the stools were soft. There had been considerable loss of weight, from 2.5 kg. in June to 1.79 kg. in November. The autopsy and study of the animal tissues with the exception of the eyes, nose, and ears were made by Dr. Howard A. McCordock, of the Department of Pathology, Washington University Medical School.

Gross postmortem changes were not unusual except for an almost complete disappearance of body fat and an opacity in the pelvis of the kidneys. There were no ulcers in the intestine. The tongue gland, sinuses, and mastoids were free from pus. There was no vitamin A in the liver. Histological study revealed no abnormalities of the gastro-intestinal tract but in the eyes, ears, sinuses, salivary glands, and kidneys there was significant epithelial metaplasia. Figures 2 to 13 show the changes in these structures. For comparison the maxillary sinus of a normal well-fed monkey is shown in figure 14 and a normal temporal bone is pictured in figure 15.

Histological examination of the preparations of the eyeballs and eyelids of monkey 125 was made by Dr. Harvey D. Lamb, of the Department of Ophthalmology, Washington University Medical School. His report follows:

Right eyeball. The peripheral part of the cornea was thickened by edema and presented considerable proliferation of corneal corpuscles and infiltration with small lymphocytes and plasma cells in the anterior half of the corneal thickness. Bowman's membrane was entirely destroyed and many capillaries were present between the corneal lamellae in the anterior half of the cornea. Over the corneal center, practically the entire corneal thickness had been destroyed and anteriorly, a thin layer of cicatricial tissue was present. The large destroyed portion of the cornea was occupied by swollen prolapsed pupillary portions of the iris and a very thick cellular mass filling the pupillary space; the latter consisted of dense numbers of small lymphocytes, plasma cells, young fibroblasts and proliferated pigmented epithelial cells from the pigmented iris epithelium. Over almost the entire posterior surface of the iris, the pigmented iris epithelium showed an intense proliferation and the formation of pigmented spindle cells, probably to become fibroblasts. This proliferated epithelial tissue was moderately and diffusely infiltrated with plasma cells. The iris was edematous and showed a small greatly varying infiltration with plasma cells.

A perforation had occurred at about the corneal center and corneal epithelium had proliferated deeply along the wound edges. The lens-capsule had been ruptured and the lens almost altogether absorbed; in places dense numbers of pus cells were present within the lens capsule and in other places thin layers of proliferated lens epithelium.

The anterior epithelium of the cornea covered its entire surface; it was generally considerably thicker than normal and on its posterior surface showed the formation of papillae. An advanced degree of xerosis was present in the corneal epithelium with generally a thick keratinized layer, keratohyaline grains and much desquamation. The basal cells in places presented edema of the cytoplasm; all but the more superficial layers of cells showed thin layers of edema between the individual cells.

Left eyeball. The cornea was uniformly thickened by edematous fluid. At and near the corneal margin, numerous capillaries were present in the anterior half of the corneal thickness, extending for a short distance into the cornea. In the same locality proliferation of the corneal corpuscles was moderate in degree.

The iris-angle contained a small group of dense numbers of pus cells.

The corneal epithelium in many places showed a flattening of all the cells except those in the basal layer; superficial layers of flattened cells were dried and desquamating without a true keratinization being noted; the basal cylinder cells were slightly edematous and all but the superficial dried cells showed generally a thin vacuolar ring about the nucleus.

Bulbar conjuctiva of both eyeballs. An advanced degree of xerosis of the covering epithelium was present with a thick keratinized layer, keratohyaline grains and much desquamation. The basal epithelial cells, in places, presented cystic degeneration of their cytoplasm.

Palpebral conjunctivae. In all of the eyelids, generally, intensive xerotic changes were noted in the covering epithelium. Thick layers of keratohyaline grains and the desquamation of thick keratinized layers were common. The covering epithelium was generally much thicker than normal with several midlying layers of polyhedral-shaped cells. Thin layers of edematous fluid were present in many places between all but the superficial flattened epithelial cells. In only a few places, the cytoplasm of the deeper lying cells were edematous. The lower part of the lower right eyelid was densely infiltrated throughout its thickness by pus cells.

Histological examination of the preparations of the sinuses and ears was made by Dr. Wm. F. Wenner, of the Department of Otolaryngology, Washington University Medical School. His report follows:

The mucous membrane lining of the maxillary sinuses and the mucous membrane of the turbinates and the nasal septum of the monkey showed marked degree of metaplasia and keratinization of the epithelium with round cell infiltration in the basement membrane. The lining membrane of the bulla of the ear was very hyperemic. The blood vessels were greatly dilated and filled with blood. The compact bones immediately underlying the membrane show evidence of decalcification. There was no evidence of cellular resorption of bone. There were no osteoclasts present.

The other monkeys on the same vitamin A-low diet lived only from 1 to 10 months before loss of appetite and loss of weight occurred. The time of year and facilities were such that it was impossible to keep them outdoors. Of all the other animals, monkey 111 remained in the best condition for the longest time. Her death, as shown in chart A, followed almost immediately after the first indications of poor appetite, soft stools, and weight loss. She always looked very healthy. She never had diarrhea. Autopsy showed a normal animal. Histological examination of the tissues also showed a perfectly normal animal in all respects but one. The epithelium of the conjunctiva of the right eyelids showed fairly extensive metaplasia from stratified columnar to stratified squamous cell epithelium but there was no keratinization. There was no vitamin A in the liver.

Monkey A_8 weighed less than 1 kg. when the experiment was begun and lived only 2 months on the deficient diet. The appetite was always poor. Autopsy showed a small normal animal with very little fat. There was as much vitamin A in the liver of this animal as in the liver of the normal control. Another of the monkeys, however, that died after only 3 months on the diet, showed no vitamin A in the liver.

As chart A shows, monkeys C_4 and C_5 lived for almost 7 months on the deficient diet. C_5 gained very little weight but C_4 almost doubled her weight before the loss of appetite and loss of weight occurred. C_4 was typical of another monkey, T_3, the only two monkeys studied showing complete loss of appetite, severe diarrhea, and abscesses on the face. Intes-

tinal ulcers were found at autopsy both in C_4 and in C_5. Histological examination showed normal eyes, kidney, and salivary glands. The uterus of C_4 was filled with pus with bacteria present. There was inflammation of the intestinal mucosa. The large intestine of C_5 showed subacute colitis with an acute ulcer. There was oedema of the submucosa. Since this animal never gained much weight, it is possible that she died from a cause other than vitamin A deficiency for both C_5 and C_4 were extremely emaciated and looked sick before death. There was no vitamin A in the liver of either of these animals.

Monkey A_5 was typical of a group of six animals who were fed varying amounts of tomato pulp after they began to show loss of weight on the deficient diet. A_5 was given 4 ounces of whole tomato daily, considerably more than was fed to the other animals, and she made the best recovery. After 4 months of ingestion of vitamin A containing food, monkey A_5 looked normal again.

Although only one of the twenty-seven monkeys on the vitamin A-low diet showed xerophthalmia and the histological changes characteristic of vitamin A deficiency, it can be concluded that the monkey (Macacus rhesus) is susceptible to the same characteristic changes due to vitamin A deficiency that have been hitherto demonstrated in man and in the albino rat. Since monkey 125 showed keratinization of the epithelial tissues of the eyes, the sinuses, the kidneys, and the salivary glands after 13 months on the vitamin A-low diet and still showed a normal gastro-intestinal tract with no ulcerative colitis and had had no severe diarrhea, it seems possible that in the monkeys of Tilden and Miller and Turner and Loew and in the three monkeys here reported, colitis was due only indirectly or else not due to the deficiency of vitamin A. The diets used by the other investigators may have been more irritating to the intestinal mucosa than the diet fed to our monkeys. We found that more than 5 per cent casein and even a daily 5 gm. of yeast caused diarrhea. Also since our source of vitamin C, tomato serum, furnished almost no vita-

min A, it was possible to feed an unlimited amount of this food. With healthy monkeys to feed and a suitable environment with sunshine and fresh air it is probable that other monkeys could live long enough on a suitable vitamin A deficient diet to show the specific keratinizing effect with no other changes.

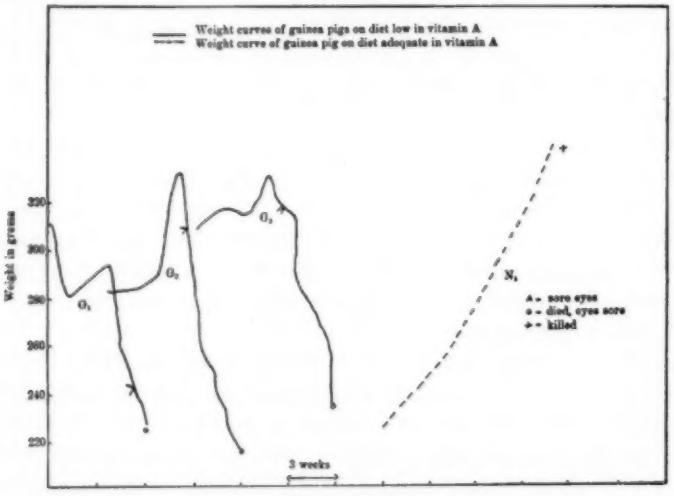

Chart B Weight curves of guinea pigs on diets with and without vitamin A.

EXPERIMENTS WITH GUINEA PIGS

Young guinea pigs weighing about 300 gm. each were housed in metal mesh cages similar to those usually used for rat breeding. The animals were offered the same vitamin A-low diet given to the monkeys. Although the guinea pigs ingested 3 to 4 ounces of tomato serum daily, their intake of monkey 'cookie' was very small. The three guinea pigs thus fed died in less than a month with no eye lesions. Three other animals were then offered the same diet with whole oats ad libitum. These animals, as chart B shows, lived for 6 to 9 weeks. During the last 2 or 3 weeks they lost weight very rapidly and developed eye lesions. The lids were dry

and scaly and the corneas were usually slightly clouded. The fur became rough and unkempt. The animals were very thin and emaciated. The stools of G_3 were bloody during the last 2 days. All of the animals shivered for 1 or 2 days before death. Histological study was made only of the eyes of these animals. A report of the microscopic findings of the eyes of guinea pig G_3, from Dr. Harvey D. Lamb, follows:

Eyeballs. The anterior epithelium of the cornea contained two to three more superficial layers of flattened epithelial cells than are present in normal eyes. Over the cornea the two or three superficial layers of flattened cells were becoming keratinized and desquamating; desquamation of a single layer or of two layers together can be seen. In places, the basal layers showed considerable cystic degeneration in the cytoplasm of the cells.

Bulbar conjunctiva. Instead of the multilayered cylindrical celled epithelium, there were present varying degrees of change to polyhedral and flattened cells in all the layers with the exception of the basal cylindrical celled layer; in places, all the layers except the basal one were changed to layers of flattened cells. The nuclei of the polyhedral and flattened cells generally showed cystic degeneration and the condensation of the chromatin into a few coarse particles. In a few places two to three superficial layers of flattened cells have become hyaline-like and dark staining. In many places, single or two superficial layers together were becoming dry and keratinized and desquamating from the surface. In most places, therefore, keratinization of the superficial flattened cells is not preceded by hyalinization of several layers together. No keratohyaline grains are seen.

Palpebral conjunctiva. In all the four eyelids, there was present the same change in varying degree from a multilayered cylindrical celled epithelium to layers of polyhedral or flattened cells with the exception of the basal cylindrical celled epithelium. Many of the nuclei of the polyhedral and flattened cells showed cystic degeneration and a concentration of the chromatin into fewer and larger granules than is normal. In many places one or two layers together of superficial flattened cells were becoming keratinized and desquamating. The condition appears to be of about the same degree in the upper as in the lower eyelids.

Figures 16 and 17 show keratinization of the epithelial tissue of the lid and cornea of guinea pig G_3.

EXPERIMENTS WITH RABBITS

Young rabbits, about 3 months old, and weighing 1.5 to 2.0 kg., were offered the same vitamin A-low diet given to the monkeys but, as with the guinea pigs, it was found that they soon died unless oats were also given. Of twelve rabbits fed the deficient diet, ten developed sore eyes in from 2 to 5 months. The other two animals died in less than 3 months before eye lesions had appeared. The rabbits gained in

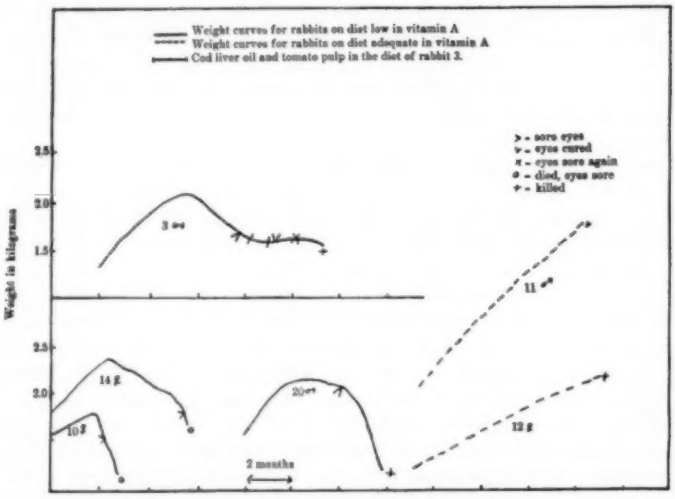

Chart C Weight curves of rabbits on diets with and without vitamin A.

weight, as shown in chart C, for a period of 2 to 4 months and appeared very normal and healthy. Usually the animals lost weight, sneezed, and had a slight discharge from the nose at about the time the lids became dry and scaly. In most of the rabbits the cornea became opaque before the animal died. When tomato pulp and cod liver oil were given to rabbit 3, the weight loss ceased and the eyes cleared, but when the vitamin A was discontinued, as is shown in chart 3, the eyes became sore again. Bitot's spots appeared in the eyes of all of the rabbits. The eyes of all of the rabbits and the

sinuses and ears of two of them were studied histologically. There were usually either keratomalacia or incipient keratomalacia of both eyes and advanced xerosis of the epithelium of the cornea, the bulbar conjunctivae, and the palpebral conjunctivae. Keratohyaline grains were noted in the bulbar conjunctiva of the left eye and corneal ulceration in the right eye of rabbit 3. Pus was found present in both eyes of all of the rabbits studied.

The mucous membrane lining of the maxillary sinuses and the mucous membrane of the turbinates of the nasal septum of the vitamin A-depleted rabbits showed a marked degree of metaplasia and keratinization of the epithelium with round cell infiltration in the basement membrane. The lining membrane of the bulla of the ear was very hyperemic. The blood vessels were greatly dilated and filled with blood. The compact bones immediately underlying the membrane showed evidence of decalcification. There was no evidence of cellular resorption of bone. There were no osteoclasts present.

The changes noted in the eyes, sinuses, and ears are shown in figures 18 to 22. Figure 23 shows the temporal bone of a normal rabbit contributed from another experiment.

Six rabbits weighing 0.8 kg. to 2.5 kg. were fed the control diet with adequate vitamin A. At first the rabbits ate very little tomato pulp, so both tomato pulp and cod liver oil were given. In about 2 months on such a diet, these control animals began to lose their fur and show stiffness in the legs. One very small rabbit weighing less than a kilogram became very crippled and lost practically all of the fur. It was suspected that cod liver oil might be responsible for this unusual condition in the rabbits and therefore it was omitted from the diet. In from 2 weeks to a month after the removal of cod liver oil from the diet the fur became normal and the legs lost some of the stiffness. The small crippled animal never recovered. These findings agree with the recent report by Wadsen, McCay, and Maynard ('33), who studied the possible relationship between cod liver oil and muscular degeneration of Herbivora fed synthetic diets. Their rabbits re-

ceiving 2 to 3 per cent of cod liver oil in a synthetic ration usually died in 30 to 40 days. Our animals did not die but our diet furnished some naturally occurring foods.

EXPERIMENTS WITH RATS

Young rats first depleted of their store of vitamin A were fed monkey 'cookie,' tomato serum, or monkey liver to test the vitamin A content of these substances. Chart D shows typical animals used for the tests. As rats M_5 and M_1 show,

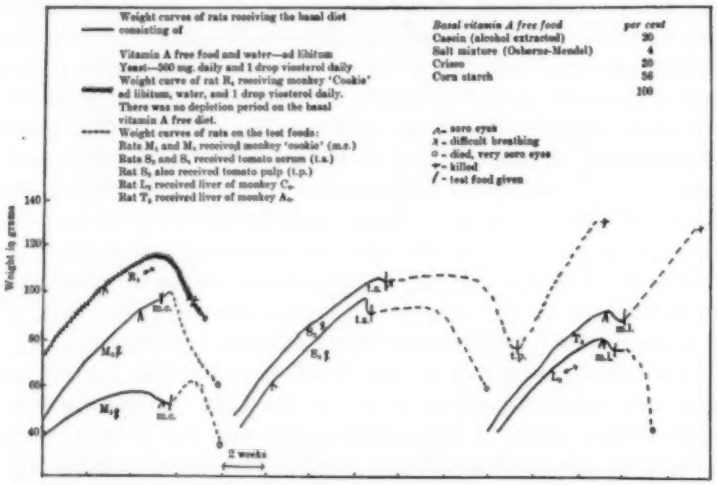

Chart D Weight curves of rats used for testing vitamin A content of monkey 'cookie,' monkey livers, tomato serum, and tomato pulp.

a temporary gain in weight occurred when monkey 'cookie' replaced the synthetic diet but the sore eyes never improved and the rats died in 2 to 3 weeks. They were in a very emaciated condition. When tomato serum was given to the depleted animals, they gained very little weight but they did usually survive for a period of 5 to 6 weeks. The condition of the eyes never improved. Neither with the monkey 'cookie' or tomato serum alone or together as a source a vitamin A did the vitamin-depleted rats ever live through the 8 weeks' test

period usually specified for determining a unit of vitamin A. It must be concluded, therefore, that the monkey diet was very low in vitamin A. The number of units of vitamin A ingested daily by the monkeys, guinea pigs, rabbits, and rats on the vitamin A-low diet was extremely small.

Young and mature rats were also fed the monkey diet with tomato pulp for added vitamin A to show the adequacy of the diet in all respects except vitamin A. Young rats thus

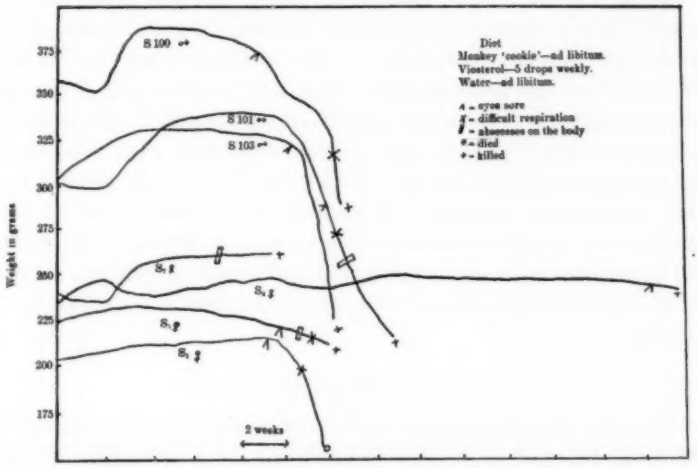

Chart E Weight curves of adult rats receiving a diet low in vitamin A.

fed gained from 2 to 3 gm. daily, which is an average normal rate of growth for a young rat. Mature females became pregnant on this diet and were successful in raising some of their young. The young, however, had very little reserve of vitamin A as is shown in the record of rat R_4, transferred immediately after weaning to the monkey diet. This animal had sore eyes in 3 weeks and was dead after 2 months on the monkey diet. The average rate of growth and the ability to reproduce on the deficient diet with added vitamin A indicates that it was adequate in all respects except vitamin A.

Adult rats were fed the same vitamin A-low diet given to the monkeys to determine how long full-grown rats could live on the monkey diet before xerophthalmia occurred. These rats usually maintained a fairly constant weight, as chart E shows, for at least 2 months and one animal was killed after 7 months with very little weight loss. Xerophthalmia occurred in 2 to 3 months in all but one animal used, and after 7 months her eyes were beginning to show some soreness. These adult animals studied usually showed difficulty in breathing about the time the eyes became sore and some of the animals had large abscesses on their bodies. One rat, S_7, was killed before the eyes became sore, for in less than 2 months her shoulder was swollen and filled with pus. Histological study was made only of the eyes of these full-grown animals. They showed advanced xerosis of the epithelium of the cornea and of the bulbar and palpebral conjunctivae. There was usually keratomalacia or incipient keratomalacia of one or both eyes with the same typical specific changes of the epithelial tissue characteristic of young rats on a vitamin A-free diet.

SUMMARY AND CONCLUSIONS

Typical xerophthalmia with keratomalacia developed in both of the eyes of one of the twenty-seven monkeys fed the diet low in vitamin A. Histological study of the tissues of this monkey showed keratinization of the epithelial structures of the eyes, the ears, the maxillary sinuses, the turbinates of the nasal septum, the salivary glands, and the kidneys. Histological study showed a normal gastro-intestinal tract. The compact bones immediately underlying the lining membrane of the bulla of the ear showed evidence of decalcification.

Rabbits, guinea pigs, and both young and adult albino rats fed the diet low in vitamin A developed xerophthalmia in most of the cases studied. Histological examination of the ears of the rabbits showed the same tissue metaplasia and bone decalcification that was observed in the ears of the depleted monkey.

The data here reported show that the monkey and the guinea pig may react to vitamin A depletion in the same specific manner as observed in the case of other animals. The storage of reserve vitamin A in the tissues and the resistance to infection during the depletion period may have been the causes for some of the apparently variable findings.

I wish to express my thanks and indebtedness to Dr. Harvey D. Lamb, of the Department of Ophthalmology, to Dr. Howard A. McCordock, of the Department of Pathology, and to Dr. Wm. F. Wenner, of the Department of Oto-Laryngology, who were kind enough to examine the microscopic tissue preparations; to Dr. Howard A. McCordock, who was kind enough to autopsy many of the monkeys, and to Doctor McCordock and to Dr. Donald M. Hetler, of the Department of Bacteriology, for the microphotography. I wish also to thank Dr. William M. James, of the Department of Ophthalmology, who devoted considerable of his time to frequent examination of the eyes of the animals under observation in this study.

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PLATES

Descriptions of photomicrographs showing histological changes in tissues of animals on a vitamin A-deficient diet. A few sections are shown from animals on a normal diet.

PLATE 1

EXPLANATION OF FIGURES

2 Section through cornea in incipient healing after sloughing of the central part of the cornea from keratomalacia, showing prolapsed iris, ingrowth of anterior epithelium of the cornea around the perforating wound edges and desquamation of superficial keratinized layers of the corneal epithelium to the right of the open wound. Monkey 125.

3 Section through one side of the cornea, showing prolapsed pupillary part of the iris to the left, organizing cellular exudative material to the right, thickening of the anterior epithelium of the cornea, thin layers of edematous fluid between the individual deeply lying epithelial cells, keratinization and desquamation of the superficial epithelial layers of the anterior corneal epithelium. Monkey 125.

4 Section through bulbular conjunctiva, showing metaplasia of the covering epithelium to stratified squamous cell form, with keratinization and desquamation of the superficial flattened cell layers, cystic degeneration of the epithelial cells of the basal layers and a downgrowth of the covering epithelium; moderate infiltration of the subepithelial connective tissue with round and pus cells. Monkey 125.

5 Section through palpebral conjunctiva, showing metaplasia of the covering epithelium to a stratified squamous cell form with keratinization and desquamation of the superficial layers of flattened cells; moderate infiltration of the subepithelial connective tissue with round cells and a few pus cells. Monkey 125.

6 Stratified squamous epithelium replacing the normal columnar lining of large duct in the submaxillary gland of monkey 125.

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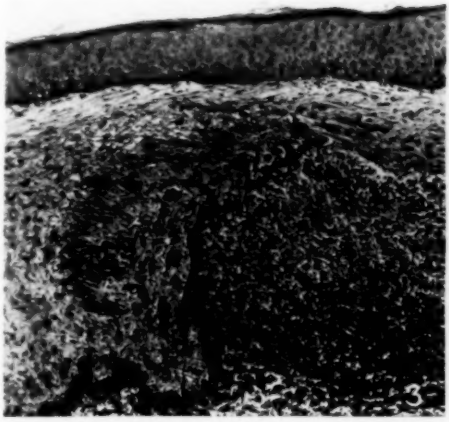
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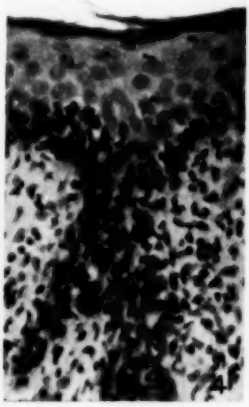

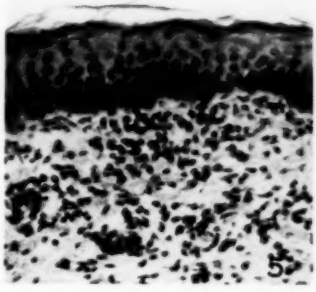
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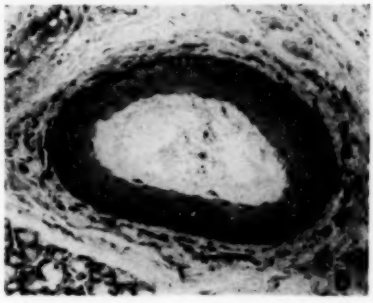


PLATE 2

EXPLANATION OF FIGURES

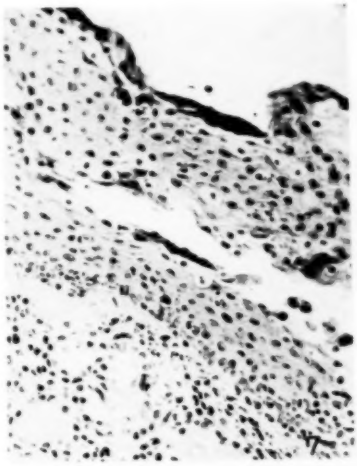
7 Mucous membrane from maxillary sinus in the region of the ostium. Section shows hyperplasia and metaplasia of the epithelium, great degree of keratinization and round cell and plasma cell infiltration in basement membrane and glandular region. Monkey 125.

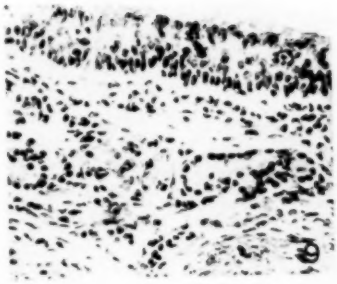
8 Mucous membrane from the lateral wall of maxillary sinus. Section shows hyperplasia of epithelium, absence of cilia, infiltration of round cells and plasma cells in basement membrane and pus in the lumen. Monkey 125.

9 Normal mucous membrane from maxillary sinus of normal monkey. Magnification the same as in figure 10.

10 Section of mucosa from posterior third of nasal septum shows keratin formation, metaplasia of epithelium and infiltration of neutrophiles, lymphocytes and plasma cells in the basement membrane with emigration of neutrophiles into the lumen passing between the epithelial cells. Monkey 125.

11 Section of mucous membrane from anterior region of middle nasal concha, showing high degree of keratinization, hyperplasia and metaplasia of epithelium and cellular infiltration. Monkey 125.

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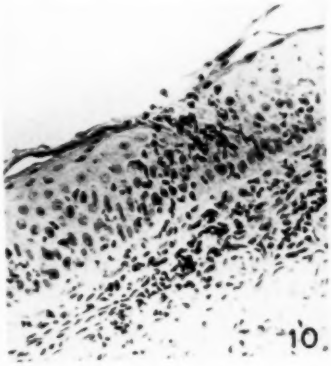
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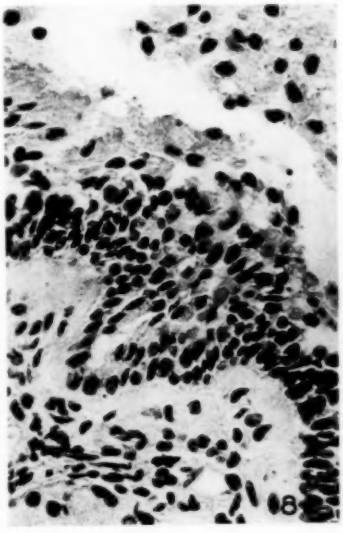
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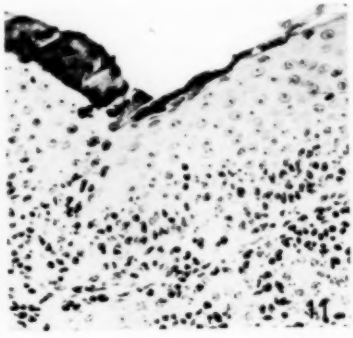
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PLATE 3

EXPLANATION OF FIGURES

12 Low-power photograph of the kidney pelvis, showing squamous cell metaplasia of the epithelium with abundant keratin production. Monkey 125.

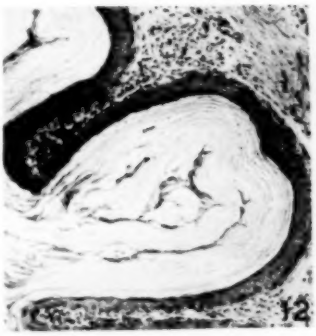
13 High-power photograph of the kidney pelvis as shown in figure 12.

14 Section from mastoid region of the temporal bone of monkey 125. The blood bessels between the epithelial cells and the periosteum are markedly enlarged. The compact bone shows evidence of decalcification.

15 Section from mastoid region of temporal bone of monkey, showing normal lining membrane and normal compact bone. Magnification same as in figure 14.

16 Section of bulba of the temporal bone of rabbit, showing enlargement and engorgement of vessels in lining membrane and decalcification of bone.

17 Section of bulba of the temporal bone of normal rabbit, showing normal lining membrane and compact bone. Magnification the same as in figure 16.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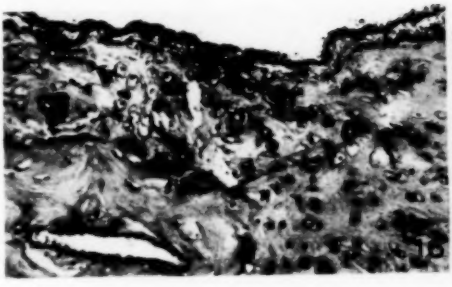
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PLATE 4

EXPLANATION OF FIGURES

18 Section through one side of rabbit's cornea in the condition of complete sloughing from keratomalacia, showing prolapse of the edematous iris.

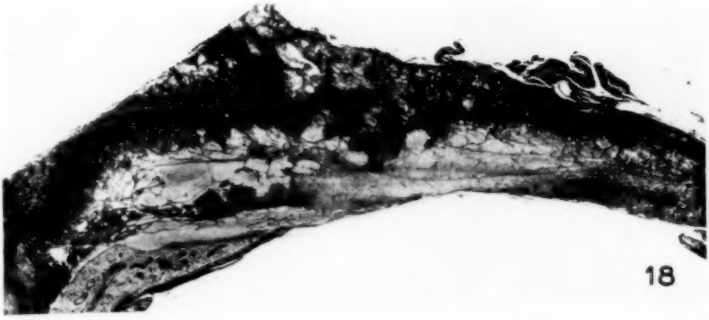
19 Section through anterior corneal lamellae of rabbit's cornea in incipient keratomalacia, showing necrosis and infiltration with pus cells of the superficial layers and a false membrane of degenerated pus cells on the surface.

20 Section through bulbar conjunctiva of a rabbit's cornea, showing metaplasia of the covering epithelium to a stratified squamous cell form with keratinization of the superficial layers of flattened cells and irregular mild cystic degeneration of deeply lying epithelial cells.

21 Mucous membrane of nasal septum of rabbit, showing metaplasia and keratinization of epithelium.

22 Section through superficial layers of cornea of guinea pig, showing an intensive degree of keratinization and desquamation of the anterior epithelium.

23 Section through the inner half of the eyelid of a guinea pig, showing the metaplasia from stratified columnar to stratified squamous cell epithelium with drying and desquamation of the superficial layers of flattened cells.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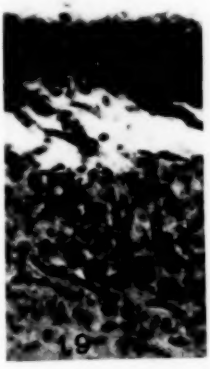
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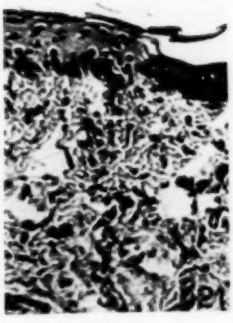
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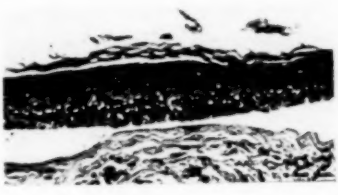
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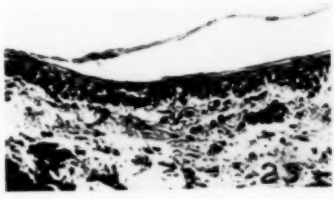
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VITAMIN D IN THE BLOOD AND MILK OF COWS FED IRRADIATED YEAST

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(Received for publication October 31, 1933)

The nutritive properties of milk have been very extensively studied. The effect of the composition of the diet on the vitamin content of milk has been the subject of numerous investigations. The dairy cow naturally presents the most advantageous subject for this work. The statements in this paper about milk, unless otherwise indicated, refer to cows' milk. The concentration of the vitamins A and D, and probably C and G, in the cow's milk can be influenced by the ration fed. It has not thus far been possible to affect the vitamin B content of cows' milk (Bechdel and Honeywell, '27; Hunt et al., '31; Kieferle, '31; and MacLeod et al., '32). Bechdel ('28) has shown that micro-organisms elaborate vitamin B in large quantities in the rumen. Consequently, supplements of vitamin B in the food may not affect the level of this vitamin in cows' milk.

The vitamin A concentration in the milk is directly influenced by the amount of this vitamin which has been or is being fed the cow, as shown by Kennedy and Dutcher ('23), Moore ('32), Fraps and Treichler ('32) and Hilton, Hauge and Wilbur ('33).

Recent work (Wachtel, '29; Steenbock et al., '30; Krause et al., '32; Hess et al., '31) has shown that the vitamin D content of milk is almost directly proportional to the vitamin D content of the food. It has been shown (Hess et al., '32) that only about 3 per cent of the vitamin D fed is secreted in

the milk, and that 27 per cent is accounted for in the feces. We were interested in determining the concentration of the vitamin D in the blood as related to the concentration of this factor in the food and milk.

EXPERIMENTAL

The material used in this study was obtained from the dairy farm of the Walker-Gordon Laboratory Company. A Holstein cow was selected from the herd which was being fed 10 ounces of 30-D yeast daily in three equal portions at 8-hour intervals. She was producing 30 pounds of milk a day. At the beginning of the experimental period, 7 A.M., June 30th, she was fed 10 ounces of 30-D yeast. This was three times the ordinary single dose. No further supplement of irradiated yeast was fed to her until after the experimental period was over. Blood samples were collected at intervals for a period of 50 hours after feeding the yeast. Milk samples were collected at 8-hour intervals for the same period. The blood was immediately centrifuged, and the blood plasma was preserved for bio-assay. Previous work (Hess et al., '32) had shown that practically all of the vitamin D in cows' blood is contained in the plasma. The per cent butter fat in the milk samples was determined, the butter fat separated and stored for bio-assay.

The bio-assays were carried out according to the method outlined by the Wisconsin Alumni Research Foundation, and the results expressed in terms of Steenbock units.

The data presented in table 1 indicate a higher rate of destruction of the vitamin D when the concentration of this factor is high in the blood. The table also illustrates very clearly the relationship between the vitamin D concentration in the blood and in the milk.

This first experiment did not show definitely how quickly after feeding the irradiated yeast the concentration of vitamin D in the blood would be at its maximum. We therefore conducted another experiment on a cow producing 30 pounds of milk daily, taken from the regular herd producing vitamin D

milk. The cow was fed her regular supplement of yeast, which in this case amounted to 1.43 ounces (one-third of the daily amount) of 70-D yeast.[1] Blood samples were taken at

TABLE 1

Data illustrating the relationship between the vitamin D in the food, the blood, and the milk

DATE	TIME	HOURS ELAPSED SINCE LAST FEEDING IRRADIATED YEAST	NUMBER OF VITAMIN D UNITS PER GRAM OF BLOOD PLASMA	NUMBER OF VITAMIN D UNITS PER QUART OF MILK PRODUCED
June 30	7 A.M.	Fed 10 ounces of 30-D yeast		
June 30	9 A.M.	2	4	
June 30	1 P.M.	6	2.5	
June 30	9 P.M.	14	1.3	
June 30	10:30 P.M.	15½		266
July 1	3 A.M.	20	1	
July 1	9 A.M.	26	0.66	
July 1	7 A.M.	24		200
July 2	9 A.M.	50	0.5	
July 2	7 A.M.	48		120

A cow which had been receiving 10 ounces of yeast daily in three portions, was given a single feeding of 10 ounces of 30-D yeast at 7 A.M. on June 30th. No further feeding of yeast was given to this cow, and her blood and milk were tested at intervals to determine the amount of vitamin D present.

TABLE 2

Influence of feeding vitamin D on the vitamin D content of blood

BLOOD SAMPLE NO.	NUMBER OF HOURS AFTER FEEDING VITAMIN D THAT BLOOD WAS TAKEN FROM COW	UNITS OF VITAMIN D PER GRAM OF BLOOD PLASMA
1	0	1.2
2	1	0.8
3	2	3
4	3	3
5	4	3

the time of feeding the yeast, and at hourly intervals thereafter for 4 hours. The blood was centrifuged, and the plasma was bio-assayed. The results of the assays are given in table 2. These data indicate that the absorption of the vita-

[1] The yeast used in these experiments was that prepared for the commercial production of vitamin D milk. In the interval between the first and second experiments the potency of this yeast was increased from 30-D to 70-D.

min D begins between 1 and 2 hours after it is ingested. The level of the vitamin D in the blood continues to decline for the first hour after feeding the irradiated yeast. From the second to the fourth hours after the feeding of the yeast the level of vitamin D in the blood remains remarkably constant.

The results shown in both tables indicate that a surprisingly high percentage of the vitamin D fed the cow is absorbed into the blood stream. Calculations based on, first, the amount of vitamin D fed; second, the increase in concentration of vitamin D in the plasma following feeding; and, third, the estimated volume of blood plasma, indicate that practically 100 per cent of the vitamin D fed appears in the blood. The amount of vitamin D which disappears from the blood cannot be accounted for either in the milk or in the feces. It is not believed that the fraction unaccounted for is stored in the body, inasmuch as the livers of cows which for months have been approximately 70,000 units of vitamin D in excess of the amount secreted in the milk or excreted in the feces, have only one unit of vitamin D per gram of fresh liver tissue at the end of this time.

DISCUSSION

Numerous studies have shown that the transfer of vitamins from the food into the milk is small in relation to the total number of units fed. Sure ('28) has shown that lactating rats require from four to five times the amount of vitamin B necessary for their own bodily needs in order to produce milk containing sufficient of this factor for the nursing young. His work indicates that probably less than 30 per cent of the vitamin B ingested is secreted in the milk. Fraps and Treichler ('32) obtained milks containing as low as 75 to 100, and as high as 2000 units of vitamin A per quart, depending on the vitamin A content of the feeds.

The work of Hess, Light, Frey and Gross ('32) indicates that about 2 to 3 per cent of the vitamin D is secreted into the milk. This figure varies slightly according to variations in the production of the cows. The vitamin D potency of the

butter fat is, roughly, inversely proportional to the per cent butter fat present in the milk, and nearly directly proportional to the amount of vitamin D fed.

The data presented in this paper indicate that practically all the vitamin D ingested is absorbed into the blood stream, although, as shown previously (Hess et al., '32), the major portion is destroyed in the body. Part is re-excreted into the intestinal tract, and is found in the feces. The data also indicate that there is a much more rapid decrease of the vitamin D in the body than had heretofore been suspected.

The secretion of vitamins A and D into milk is similar in one respect, namely, that in the neighborhood of 2 to 3 per cent of the ingested vitamins are secreted into the milk. They differ, however, in that vitamin A appears to be much more stable in the body than vitamin D, and much of the excess vitamin A fed is stored in the liver, where it may act as a reserve supply when the foods contributing this factor are lacking in the ration (Emmett et al., '32; Moore, '31).

Our results would seem to indicate that more work should be undertaken to show how long an excess above that of a normal therapeutic dose of vitamin D will remain in the body. Accurate data would be valuable as an index of the importance of regular supplies of this vitamin. This method of studying the course of the metabolism of vitamin D may also afford an explanation of the differences in therapeutic value which various sources of vitamin D have on different species. Since vitamin D disappears so rapidly from the blood stream, due probably to the fact that it is destroyed, it is not unlikely that substances present in the blood stream or in the vitamin D carrier may influence its rate of disappearance and consequently its effectiveness.

CONCLUSIONS

1. The vitamin D present in irradiated yeast is practically completely absorbed.

2. Evidence points to a rapid disappearance of vitamin D from the blood stream at a maximum rate of approximately 10 per cent per hour followed by a decline in the rate of destruction as the concentration decreases.

3. The concentration of vitamin D in the blood plasma governs the concentration of this factor in the milk.

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THE USE OF THE METHOD OF PARTIAL REGRESSION IN THE ANALYSIS OF COMPARATIVE FEEDING TRIAL DATA,[1] PART I

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(Received for publication November 4, 1933)

1. OBJECT OF STUDY

From the standpoint of the clarity of interpretation of results, an ideal feeding trial intended to evaluate feeds or rations would be one in which all the animals were alike at the start and consumed equal quantities of feed during the test. This condition is never attained in actual practice, and various procedures are employed to correct the results for the effects which the variations in the experimental animals have on them. Through careful allotment, individual differences in age, sex, breeding, thrift, condition, etc., are equalized as far as possible between experimental lots. This appears at the present time to be the only practicable method of dealing with those factors which do not lend themselves to accurate description in numerical terms. Its efficiency will depend in a large measure on the skill of the operator.

Initial weight and feed consumption, however, are usually treated differently. Differences in the initial weights of the animals are first equalized as well as possible between lots by the allotment procedure and then further corrected for in

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the analysis of the trial by subtracting them from the final weights to obtain the gains in live weight. These gains, rather than the actual weights themselves, are generally employed in interpreting the results of the trial. Then corrections for varying feed consumption for purposes of analysis and interpretation of the test have been attempted by calculating a simple gain-feed ratio.

This method of equalizing by allotment the final weights for differences in initial weights is restricted in use to the original assortment of animals into feeding groups, which in many cases may be at the start of a preliminary feeding period. Nor is the calculation of the gain always the most efficient procedure, since, if the correlation between initial and final weights is low or negative, the standard deviation of the gains will be larger than that of the final weight. ($s_D^2 = s_I^2 + s_F^2 - 2\,r\,s_I\,s_F$.)

The use of the gain-feed ratio as the basis for statistical analysis of feeding trial data appears to demand two assumptions: a) that gain is independent of initial weight and, b) that gain is directly proportional to feed intake.

That gain is not independent of initial weight is shown by correlation studies presented later in this paper. Furthermore, previous work by one of us (Crampton, '33) has shown that gains are not directly proportional to feed intake, presumably since a certain amount of feed must be supplied a pig for maintenance alone. Consequently, even if the correlation between gain and feed eaten be perfect so that the gain is uniquely defined as a linear function of the feed, that function will not be of the form $g = b\,f$ but rather $g = a + b\,f$. The gain per 100 pounds feed then becomes:

$$\frac{100\,(a + b\,f)}{f}$$

which is not a constant but a variable quantity tending to the limit b as f is increased indefinitely. As in practice f cannot be increased indefinitely but assumes various finite values, ratios obtained will be subject to systematic errors.

When this calculation is made for each pig of the trial, a statistical analysis of the array of ratios obtained will include, in addition to the experimental error, a systematic error which may be of sufficient magnitude seriously to distort the result, and to vitiate the estimate of experimental error and hence any test of significance.

This paper reports a study of the relationships which exist between the initial weights, feed consumption and live weight gains of swine. The use, in the interpretation of feeding data, of the method of partial regression as a means of correcting live weight gains for the effects of differences in the initial weights of the experimental animals and of varying feed consumption during the feeding period is also discussed.

2. OBSERVATIONAL DATA

The material studied consists of results of a fairly extensive series of feeding trials at Macdonald College in which the rations and treatment of the pigs were comparable. Individual, hand feeding was employed in all cases and individual feed records are available for each animal. Pigs were weaned at approximately 60 days of age and went onto test immediately.

The data thus available were divided into four series, according to the class of pig and its treatment:

Series A. Pigs fed for 30 days, beginning at weaning or at approximately 60 days of age.

Series B. Pigs fed for 60 days, beginning 30 days after weaning or at about 90 days of age.

Series C. Pigs fed for 30 days, beginning 90 days after weaning or at about 150 days of age.

Series D. A combined growing and finishing period. It covered 75 days of feeding, and included one change in ration at the end of 30 days at which time the proportion of protein supplement was reduced from 20 per cent to 10 per cent of the ration.

The rations used were as follows:

	Basic feeds [1] Per cent	*Protein-mineral supplement* [2] Per cent
Series A, 30 days	70	30
Series B, 60 days	80	20
Series C, 30 days	90	10
Series D { 30 days	80	20
Series D { 45 days	90	10

[1] Basic feeds consisted of corn, barley, hominy, wheat or mixtures of them.

[2] Standard Macdonald protein-mineral supplement—40 tankage, 20 fish meal, 20 linseed oilmeal, 10 bone meal, 7.6 limestone, 2 salt, 0.4 ferric oxide.

Table 1 gives a summary of the analysis of the variance and covariance of the initial weights, feed intake and gains of the animals in the various classes.

It should be mentioned that while the pigs in series A were originally allotted with due consideration of initial weight, no such sorting was made in any of the other series, since these subsequent periods were but parts of longer trials, the original allotment for which took place at the beginning of the respective weanling periods. This, in addition to the fact that differences in weights between pigs of the same ages would naturally tend to be greater in older than in younger pigs, accounts for the differences between the four series in variation of initial weights.

3. STATISTICAL ANALYSIS AND RESULTS

As will be seen from table 1, there are, excepting initial weight and gain in series D, highly significant correlations between initial weights, feed eaten, and gains in weight made by the pigs.

These relationships may, moreover, be utilized to correct the observed gains for differences in initial weight and feed consumption, and thus markedly to reduce the variability of the results, without introducing those errors inherent in the gain-feed ratio, to which attention has been directed.

Before proceeding to a consideration of the practical application of the method, however, it may be of interest to examine somewhat more closely the nature of the relation-

ship between initial weight, feed consumption, and gains, in the four series. The crude values of the coefficients of correlation between gain and feed intake and between gain and

TABLE 1

Standard deviations of and correlations between initial weight, feed eaten and gain in live weight of swine of different age groups

VARIABLES	MEAN	VARIANCE OR CO-VARIANCE	S.D.	C.V.	r	r NECESSARY FOR P = .01
Series A (N = 167)						
Initial weight, pounds (x_1)	28.6	42.59	6.53	22.83	...	...
Feed eaten, pounds (x_2)	106.5	408.66	20.22	18.99	...	...
Gain in weight, pounds (y_1)	38.3	67.08	8.19	21.38	...	...
Initial weight × gain ($x_1 y_1$)	....	23.49	....	....	0.44	0.20
Initial weight × feed ($x_1 x_2$)	....	80.39	....	....	0.61	0.20
Feed eaten × gain ($x_2 y_1$)	....	147.90	....	....	0.89	0.20
Series B (N = 69)						
Initial weight, pounds (x_1)	64.1	155.51	12.47	19.45	...	...
Feed eaten, pounds (x_2)	391.7	2411.22	49.10	12.54	...	...
Gain in weight, pounds (y_1)	94.6	227.47	15.08	15.94	...	...
Initial weight × gain ($x_1 y_1$)	....	71.24	....	....	0.38	0.30
Initial weight × feed ($x_1 x_2$)	....	402.30	....	....	0.66	0.30
Feed eaten × gain ($x_2 y_1$)	....	498.06	....	....	0.67	0.30
Series C (N = 89)						
Initial weight, pounds (x_1)	155.5	471.40	27.71	13.96	...	...
Feed eaten, pounds (x_2)	240.8	802.25	28.32	11.76	...	...
Gain in weight, pounds (y_1)	51.3	86.65	9.31	18.14	...	...
Initial weight × gain ($x_1 y_1$)	....	100.19	....	....	0.50	0.27
Initial weight × feed ($x_1 x_2$)	....	344.23	....	....	0.56	0.27
Feed eaten × gain ($x_2 y_1$)	....	145.80	....	....	0.55	0.27
Series D (N = 48)						
Initial weight, pounds (x_1)	67.6	129.30	11.37	16.81	...	...
Feed eaten, pounds (x_2)	514.8	4564.75	67.56	13.12	...	...
Gain in weight, pounds (y_1)	125.5	247.53	15.73	12.53	...	...
Initial weight × gain ($x_1 y_1$)	....	36.55	....	....	0.20	0.35
Initial weight × feed ($x_1 x_2$)	....	575.41	....	....	0.75	0.35
Feed eaten × gain ($x_2 y_1$)	....	624.79	....	....	0.59	0.35

initial weight given in table 1 suffer from the defect that initial weight and feed consumption are themselves correlated. In order to obtain an estimate of the independent effect

of each, the method of partial correlation must be employed. It will be advantageous to calculate the partial regression coefficients rather than the partial correlation coefficients, for the former are not only of more interest in themselves, but may be used directly to reduce the variability of the gains.

We may proceed to the calculation of the partial regression coefficients for each series by solution of the following simultaneous equations[3]:

$$b_1 S(x_1^2) + b_2 S(x_1 x_2) = S(x_1 y)$$
$$b_1 S(x_1 x_2) + b_2 S(x_2^2) = S(x_2 y)$$

where b_1 and b_2 are the partial regression coefficients of gain on initial weight and feed consumption, respectively; and $x_1 =$ initial weight, $x_2 =$ feed eaten, $y =$ gain. S stands for the summation of all sets of observations corrected to their respective means, i.e., $S(x_1^2) = S(x_1 - \bar{x}_1)^2$, etc.

Since to test the significance of the regression coefficients we shall need to know the values of c_{11} and c_{22},[4] it will be somewhat simpler first to obtain the c values by writing the equations:

$$b_1 S(x_1^2) + b_2 S(x_1 x_2) = 1, 0$$
$$b_1 S(x_1 x_2) + b_2 S(x_2^2) = 0, 1$$

and the solutions of the two sets of equations as—

$$b_1 = c_{11}, c_{12}$$
$$b_2 = c_{21}, c_{22}$$

The regression coefficients may then be found by substituting in the formulas:

$$b_1 = c_{11} S(x_1 y) + c_{12} S(x_2 y)$$
$$b_2 = c_{21} S(x_1 y) + c_{22} S(x_2 y)$$

The results thus obtained from series A, B, C and D are given in table 2.

From table 2 some interesting inferences may be drawn. First, it should be noted that all of the partial regression coefficients of gain on feed consumption are highly significant,

[3] See Fisher, R. A., Statistical methods for research workers, p. 132, 3rd ed.
[4] See Fisher, R. A., Statistical methods for research workers, section 29.

t for P = .01 with n greater than 30, being given by Fisher as 2.58. The decreasing rate at which gains are made per unit of feed eaten with advancing age of pig is also clearly shown.

The effects of initial weight on gains are perhaps surprising at first glance. It would seem that for weanling pigs and for trials involving young pigs fed to or nearly to market weight, the calculation of gains does not efficiently correct the data for the effects of differences between pigs in initial weight. With older pigs the effects of differences in initial weight on gains are of less importance. It appears that with young growing pigs the larger the pig at the start of the feeding the smaller the gain.

TABLE 2

Partial regression coefficients, and their standard errors, of gains on initial weight (b_1) and feed eaten (b_2)

SERIES	N	INITIAL WEIGHT (b)				FEED EATEN (b)			
		b_1	Standard error ($s\sqrt{c_{11}}$)	$t = \frac{b_1}{s\sqrt{c_{11}}}$	P	b_2	Standard error ($s\sqrt{c_{22}}$)	$t = \frac{b_2}{s\sqrt{c_{22}}}$	P
A	167	—0.209	0.053	3.94	<0.01	0.403	0.018	22.77	<0.01
B	69	—0.134	0.145	0.92	0.35	0.229	0.032	7.17	<0.01
C	89	0.116	0.272	0.41	0.68	0.132	0.034	3.88	<0.01
D	48	—0.744	0.222	3.35	<0.01	0.230	0.037	6.17	<0.01

The results with series D are particularly interesting, since this group corresponds closely to a common type of hog feeding trial in which young pigs averaging from 60 to 70 pounds are fed to market weight. Here it seems evident that initial weight is of pronounced importance in the gains made by the pigs. Given pigs of the same age and eating equal amounts of feed, each extra ¾ pound of weight at the beginning of the trial results on the average in a penalty of 1 pound of gain at the end of a 75-day feeding period. This may probably be partially accounted for by the fact that extra weight on a young pig of, say, 90 days of age usually implies that the animal is carrying considerable fat and hence not in condition to make most rapid gains for feed eaten. Furthermore, larger

pigs are likely also to be more mature and hence to require more feed for a unit of gain.

By reference to table 1, it appears that in series D initial weight is less closely related to gain than to feed consumption. However, as indicated on page 117, the apparent correlation between any two of these factors is affected by the correlation of both with the third factor. The results given in table 2 indicate that there is, in fact, a significant relation between initial weight and gain. The variability of the initial weights of the pigs in this series is by no means abnormal, being measured by a standard error of about 17 per cent which will compare favorably with that of average feeding trials. (An average of the standard deviations of initial weights reported by Mitchell and Grindley ('13) for some twenty lots of hogs in which the initial weights were comparable to those of this study, i.e., 60 to 70 pounds, is approximately 13.0, or very nearly 20 per cent.)

It is evident that, except possibly during the fattening period, i.e., when the animals have passed the period of rapid growth, both feed and initial weight should be considered for their effects on gains. To omit the factor of initial weight, which is done where the gain-feed ratio is used, is certainly to be questioned. This would be particularly true in the case of a feeding period analyzed by parts.

As an example, take the case in which two or more rations for weanling pigs are to be studied. The experimental difference, such as the addition to one ration of cod liver oil, might apply only during a short period at the start of the trial, as in series A, and then for the remainder of the feeding to market weight the comparative lots be carried on identical rations in order to learn if the cod liver oil fed during the weanling period affected the subsequent progress of the pigs. Obviously, it would be impossible to reallot the pigs at the end of the cod liver oil feeding sub-period, and hence the initial weights of the pigs for the subsequent period would likely be much more variable and poorly distributed than ideal allotment would call for. Analysis of the gains made during

such subsequent periods, without regard to the effects of varying initial weights, would be less accurate than if this related factor had been taken into account.

4. APPLICATION TO REDUCTION OF VARIANCE

The variance of the individual gains furnishes a direct measure of the precision of any feeding trial, for the significance of experimental differences can only be established by contrasting them with the differences existing among animals treated alike. It has been shown in section 3, however, that, in the present instance at least, differences in initial weight and in feed consumption contribute to this variance; and the regression equation—

$$Y = \bar{y} + b_1 (x_1 - \bar{x}_1) + b_2 (x_2 - \bar{x}_2)$$

may be used to free the observed gains of fluctuations attributable to the above factors. The increase in precision thus effected may be shown by a comparison of variance of the raw gains (y) with that of the corrected gains (Y). The sum of the squares of $(y - Y)$ is easily obtained, since—

$$S(y - Y)^2 = S(y^2) - b_1 S(x_1 y) - b_2 S(x_2 y)$$

and to obtain the variance, therefore, we calculate:

$$s^2 = \frac{1}{n' - p - 1} S(y - Y)^2$$

n being the number of observations, and p the number of coefficients calculated therefrom—in this case two.

Table 3 gives for each series the sum of squares, variance and standard error of the raw gains (y) and the gains corrected by the regression coefficients.

TABLE 3

Analysis of variance of gains

SERIES	RAW GAINS				GAINS CORRECTED BY REGRESSION FOR INITIAL WEIGHT AND FEED INTAKE				RELATIVE PRECISION
	D/F	Sum of squares	Variance	Standard error	D/F	Sum of squares	Variance	Standard error	
A	166	11134.6	67.08	8.19	164	2054.2	12.53	3.54	5.4
B	68	15468.2	227.47	15.08	66	8365.8	126.76	11.26	1.8
C	88	7625.2	86.65	9.31	86	6957.3	80.90	8.99	1.1
D	47	11634.1	247.53	15.73	45	6139.8	136.44	11.68	1.8

It is very evident from this table that, except in the case of the fattening pigs (series C), a definite increase in precision is obtained in an analysis of gains by correcting them for the effects of varying initial weights and feed intake. In the case of weanling pigs, for instance, it would be necessary to use fifty-four pigs to obtain as reliable an average gain as could be obtained with ten pigs with gains corrected for these two related factors.

It may be pointed out that this method of analysis is not confined to the items of initial weight, feed intake and gains, but is equally applicable to any of the correlated variables of a trial which can be numerically expressed. For instance, it is the practice in certain cases to remove pigs from test at a predetermined final weight instead of after a fixed length of feeding period, and to use the days on feed as the basis of the analysis. In such cases, the number of days fed would become the dependent variate to be corrected for the effects of initial weight of pigs and feed consumption.

Another possibility which seems to offer certain advantages is to dispense entirely with the gain figure, basing the statistical analysis on the final weights of the pigs corrected by partial regression for effects of variations in initial weight and feed consumption.

ANALYSIS OF COMPARATIVE FEEDING TRIALS

In the present communication we have been concerned mainly to demonstrate the relationship existing between gains and initial weight and feed consumption of swine fed on the same ration, and to indicate the manner in which this relationship may be used to reduce the variance of the gains. The method is, however, also applicable to comparative feeding trials, in which the animals do not all receive the same kind of treatment or ration, but are divided into two or more differently treated lots. In such cases the statistical procedure known as the analysis of covariance enables the regression coefficients to be estimated from the variance and covariance within lots, and provides a means not only of reducing the

variance of the results, but also of correcting the observed differences in gains between lots for the disturbing effects of differences in average initial weight and feed consumption.

SUMMARY

Statistical analysis by the method of partial regression has been employed to determine the relationship between gains and initial weight and feed consumption of swine of four different age groups. Highly significant relationships between gains and feed consumption are exhibited by all groups, the increase in gain due to each additional unit of feed consumed decreasing with advancing age of pig. In the case of weanling pigs and young pigs fed to market weight, the gain made is also dependent upon the initial weight of the pig.

The gain-feed ratio commonly employed does not efficiently correct the observed final weights for variations in initial weight and feed intake. A satisfactory correction may, however, be made by means of the regression coefficients, markedly reducing the variance of the results without introducing errors inherent in the gain-feed ratio. This procedure is also applicable to comparative feeding trials, in which the animals are divided into two or more differently treated lots.

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IRON AND COPPER RETENTIONS IN YOUNG CHILDREN

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(Received for publication October 30, 1933)

Although iron is an essential constituent of animal protoplasm, the larger proportion existing in the hemoglobin of the blood, there is a paucity of data in the literature regarding the actual amount required by the human organism, especially by children. In fact, only two such studies with children of preschool age were found in the recent literature. Rose and co-workers ('30) have reported a study with a 31-month-old child in which a negative balance was obtained with an ingestion of 0.33 mg. of iron per kilogram, and Leichsenring and Flor ('32) working with four normal children between 3 and 6 years of age obtained retentions of 0.07 and 0.18 mg. per kilogram at ingestion levels of 0.19 and 0.36 mg., respectively. The iron excretion, 0.12 mg. per kilogram, at the lower ingestion level the latter authors interpreted as an index of the maintenance requirement, whereas the retention, 0.2 mg. at the higher level, was the amount needed for growth. The total minimum requirement, therefore, was estimated to be 0.32 mg. per kilogram. To this the authors add 50 per cent as a margin of safety to cover individual differences.

According to recent investigations (Hart, '28; Elden, '28; and Kraus, '29) iron alone cannot effect a formation of hemoglobin; small amounts of copper must be present to stimulate the physiological processes. Therefore, studies of the iron requirements would seem to be incomplete without information regarding the copper requirements.

The amount of copper needed by the human organism apparently is not known. A search of the literature reveals no studies of a quantitative nature, with the exception of determinations of the copper content of various human tissues (Bodansky, '21; Morrison, '30). The concentration of copper in the blood in relation to that of iron might be expected to give some hint as to the relative amounts of these elements which should be ingested. From the data available, the ratio of iron to copper in the blood of different animals was found to vary from 34:1 for the cow (McHargue, '28) to 740:1 for the rat (Lindow, '29). Elvehjem, Steenbock, and Hart ('29) fed a ration to rats in which the ratio of iron to copper was 50:1, a much larger amount of copper than would seem necessary if the concentration of iron and copper in rat blood were taken as a criterion. In children the iron-copper ratio of the blood has been estimated to be 300:1 (Sobel, '33; Gorter, '31). Whether the human organism requires more or less copper in relation to the amount of iron than the rat remains to be determined.

EXPERIMENTAL PROCEDURE

In the investigation, iron and copper retentions have been determined in eight normal children, three girls and five boys ranging in age from 3 to 6 years. From one to three studies were made with each child. Two types of diets were fed, varying in the choice of foods, but containing relatively slight differences in the amounts of copper and iron. Although it would have been desirable to study the retentions on diets containing a wider range of iron and copper, the close agreement was necessitated by the fact that the diets were planned to meet the needs of another study. The experimental period consisted of 8 days, the first 3 days for adjusting the children to the diet, and the last 5, the metabolism period. During the study the children, who were under constant supervision, received accurately weighed diets. Urine and feces were collected quantitatively.

The two types of diets (samples of which are given in table 1) differed in that one contained cereal, meat, eggs, vegetables, and fruits; whereas the other although similar, contained a larger amount of cereal foods and no meat. The milk used was either pasteurized or evaporated.

The foods used were purchased in quantities sufficient to last the entire period, the perishable materials being kept in a low-temperature refrigerator. In preparing the diets, the cereals and sugar were weighed dry. At the beginning of each experimental period, potatoes and carrots were pared, cut into inch cubes, and kept in distilled water. In this way a more nearly uniform material was obtained. When these were to be used, they were drained, dried with a towel, weighed and so cooked that there was no excess liquid to be discarded at the end of the process. Each child's food was prepared in separate containers. Prunes and apples for the entire period were cooked in distilled water, sieved to insure a uniform mixture, and weighed after cooking. Bananas were taken from a single stalk. The meat, ground round steak, was weighed raw and cooked in porcelain ramekins in which it was served. The bread for a given child was cut from the same loaf. Sufficient orange juice, as well as canned tomato, was prepared at the beginning of the period. Eggs for the period of study were thoroughly beaten and a portion taken for the day's serving. When evaporated milk was used, one large can sufficed for the 8-day study. The pasteurized milk was obtained fresh each morning and an aliquot from each day's supply was taken for analysis. All foods for the children, as well as the food to be analyzed, were cooked in aluminum or glazed porcelain vessels. The food prepared for analysis was from the same supply as that served the children. Distilled water was used in all cases for cooking and drinking.

Since the milk for analysis was taken from the supply served to the children in a given group, it was necessary to make only one determination for the three balance studies. This was measured into porcelain crucibles, dried in a Freas

TABLE 1

Typical diets of children during experimental periods

NAME	DATE	DIET [1]	PRUNES	OATMEAL	MILK	BREAD, WHOLE WHEAT	ORANGE JUICE	POTATO	MEAT	TOMATO	APPLESAUCE	EGG	CARROTS	BANANAS	RALSTON	SUGAR	BUTTER	COD LIVER OIL
			gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
A.C.	2/3	A	60	12.6	487.5	65	120	100	50	110	90	75	70	90	..	27	28	7.2
A.C.	2/11	B	60	12.6	487.5	65	120	100	50	110	90	75	70	90	..	27	28	7.2
A.C.	3/27	C	55	20.0	487.5	100	120	130	..	110	85	60	..	120	20	18	14	7.2
D.B.	4/7	D	40	20.0	487.5	80	120	110	..	110	70	60	..	110	20	15	11	7.2

[1] Diet A. Meat with pasteurized milk.
Diet B. Meat with evaporated milk.
Diet C. High cereal with pasteurized milk.
Diet D. High cereal with evaporated milk.

oven at a temperature of 60°C., and ashed in an electric muffle furnace, 50 cc. being taken for the iron determinations and 250 cc. for copper.

Similarly, analyses were made on composite samples of the day's supply of food prepared in the same way as that fed. This was placed in a large porcelain evaporating dish, thoroughly mixed with acid alcohol, evaporated to dryness on a steam bath, brought to constant weight in a drying oven, ground in a porcelain mortar, and stored in a desiccator until such time as analyses could be made. Aliquot samples of the dried foods were likewise ashed in porcelain crucibles, 5 gm. being taken for the determinations of iron and 20 gm. for copper.

Stools for the 5-day period, marked with 0.2 gm. of carmine, were treated in the same manner as the food, 2 and 5 gm. of the dried material being taken for the copper and iron determinations respectively.

The daily urinary excretions were made up to a given volume with distilled water. Aliquots were pooled and the analyses made of the composite sample, 150 cc. to 270 cc. being used for iron and 1000 cc. to 2000 cc. for copper. The measured amounts were then dried in pyrex beakers on a steam bath, the residues dissolved in concentrated nitric acid and transferred quantitatively to porcelain evaporating dishes, which were heated slowly over an open flame until the nitric acid was driven off and the material completely ashed.

Iron was determined by the method of Elvehjem and Hart ('26) and copper according to the analytical procedures of Elvehjem and Lindow ('29). All precautions suggested by the authors were carefully followed: Distilled water was redistilled from glassware; porcelainware was treated with alcoholic sodium acetate, ignited, and subsequently extracted with 1:1 HCl for at least 4 days. Determinations were made in triplicate with the exception of the copper analysis of urine for which there was an insufficient amount of material. Duplicate determinations were therefore made in all but three cases, in which there was enough material for only one.

Analyses of food, feces, or milk which seemed questionable were repeated.

In order to test the reliability of the technics employed, known amounts of iron and copper were added to given samples of food and feces. The percentage recovery, based on this procedure, was found to be 101.1 per cent in the iron

TABLE 2

Tests for accuracy of method

IRON IN ORIGINAL SAMPLE	IRON ADDED	IRON RECOVERED	IRON RECOVERED
		Iron	
Mg.	*Mg.*	*Mg.*	*Per cent*
0.7598	0.1000	0.8609	101.1
0.7890	0.1000	0.8922	103.2
0.2442	0.1000	0.3431	98.9
		Average:	101.1

COPPER IN ORIGINAL SAMPLE	COPPER ADDED	COPPER RECOVERED	COPPER RECOVERED
		Copper	
Mg.	*Mg.*	*Mg.*	*Per cent*
0.2564	0.1000	0.3529	96.5
.....	0.1000	0.1000	100.0
0.0870	0.1000	0.1832	96.2
		Average:	96.2

analyses and 97.6 per cent for copper (table 2). Blank determinations were made on reagents each time new solutions were prepared, and corrections made on each iron analysis accordingly. Analysis of the reagents used in the copper determinations gave negative results. The distilled water used for drinking and cooking, as well as that used for diluting the urine, contained no copper, and only a faint suggestion of iron, in 800 cc.—too little to test.

RESULTS AND COMMENTS

Iron

Both the iron and copper of the food differed somewhat from the theoretical amounts estimated from analyses reported for iron by Peterson and Elvehjem ('28) and for copper by Lindow, Elvehjem and Peterson ('29). Wide variations in the mineral content of plant foods apparently are to be expected, depending upon conditions under which they are raised (Remington, '30).

The variation in the iron and copper contents of milk was particularly noticeable. The milk analyzed contained from 1.24 to 2.22 mg. of iron per liter, and 0.22 to 0.76 mg. of copper—results which come within the range of values reported by other investigators. For example, Rose found that milk furnished about 0.60 mg. of iron per liter; Leichsenring and Flor reported 1.80 mg., while Peterson and Elvehjem gave the average iron content of milk as 2.24 mg. per liter.

The normal copper content of milk has been reported by Lindow, Elvehjem and Peterson as 0.15 mg. per liter; Supplee and Bellis ('22), using a different method of analysis, found 0.2 to 0.8 mg. per liter; and Quam and Hellwig ('28) obtained results varying from 0.26 to 0.52 mg. The copper contents of the three samples of evaporated milk used in this study were considerably higher (0.33, 0.62, and 0.76 mg. per liter) than the values obtained for the pasteurized milk (0.22, 0.26, and 0.35 mg. per liter). Whether the larger amount was present in the original milk or was due to contamination during the manufacturing process is not clear.[1] Rice and Miscall ('23) and Rice ('26) have shown that copper is dissolved from the vacuum pan during the condensing process in the manufacture of condensed and powdered milk, thus increasing the quantity of copper in these products to a considerable extent. The evaporated milk used in the children's diets, according to a statement by the manufacturers, was condensed under vacuum in copper pans.

[1] Since this manuscript was completed, the report by Stein and Lewis has confirmed our findings regarding the higher copper content of evaporated milk. J. Nutrition, 1933, vol. 5, p. 465.

The iron intake of the children studied ranged from 0.57 to 0.75 mg. per kilogram and from 0.60 to 0.75 mg. per 100 Calories of food. These amounts approximate the requirement of 0.61 mg. proposed by Rose et al., but exceed the suggested standard given by Leichsenring and Flor. The ingestions, estimated on the 100 Calorie basis, conform rather closely to the standard of the latter workers, but are somewhat lower than the amounts advocated by Rose; the caloric ingestions of the children in the studies, however, were higher.

From 94 to 97 per cent of the iron excreted was found in the feces. The amount in the urine varied from 0.22 to 0.55 mg. per day. No outstanding relationship between intake and urinary output was noted.

The iron retentions of the children studied varied from 0.12 to 0.27 mg. per kilogram (average 0.18 mg.). At the same ingestion level, A.C. retained 0.24 mg. per kilogram during the first period (table 3) and only 0.15 mg. during the subsequent period: R.H. also retained more during the first metabolism period, whereas B. C. retained practically the same amounts during all three periods. The explanation for the higher retentions during the first metabolism periods may be previous food conditions. These children lived in a children's home where food conditions were good, but perhaps not optimal. Undernourished children are known to retain larger amounts of essential elements than those who are well stocked (Wang, '29; Stearns, '21; and Daniels, '33). The iron retentions of D. B. and D. P. (diet D) were low (0.13 and 0.12 mg. per kilogram) perhaps because they needed less. These two were the only children of the group who came from private homes. Moreover, during the preceding 2 years they frequently had served as subjects for nutrition studies and had received what are believed to be optimum diets.

In the present study, the highest iron retentions found were 0.24 and 0.25 mg. with ingestions of 0.59, 0.65, 0.75 mg. per kilogram respectively, retentions which are no higher than one of those obtained by Leichsenring and Flor at a much

TABLE 3

Average daily intake, excretion, and retention of iron

NAME	DATE	WEIGHT	ENERGY INTAKE	IRON INTAKE					IRON EXCRETION			IRON RETENTION	
				Milk	Food	Total	Per kg.	Per 100 Cals.	Urine	Feces	Total	Total	Per kg.
Diet A. Meat with pasteurized milk													
		Kg.	*Cals.*	*Mg.*	*Mg.*	*Mg.*	*Mg.*	*Mg.*	*Mg.*	*Mg.*	*Mg.*	*Mg.*	*Mg.*
A.C.	2/3	18.0	1677	0.59	10.02	10.61	0.59	0.63	0.29	6.03	6.32	4.29	0.24
B.C.	2/3	13.5	1272	0.59	8.11	8.70	0.64	0.68	0.33	5.78	6.11	2.59	0.19
E.I.	2/3	22.6	1974	0.59	12.41	13.00	0.58	0.66	0.34	8.62	8.96	4.04	0.18
L.T.	3/5	15.0	1613	0.77	8.90	9.67	0.65	0.60	0.39	5.35	5.74	3.93	0.25
R.H.	3/5	18.1	1628	0.77	9.73	10.50	0.58	0.64	0.55	7.71	8.26	2.24	0.12
Diet B. Meat with evaporated milk													
A.C.	2/11	18.3	1677	0.70	10.58	11.28	0.62	0.67	0.33	8.26	8.59	2.69	0.15
B.C.	2/11	13.7	1272	0.70	7.52	8.22	0.60	0.65	0.34	5.35	5.69	2.53	0.19
E.I.	2/11	23.0	1974	0.70	12.70	13.40	0.59	0.68	0.35	9.88	10.23	3.17	0.14
G.O.	2/25	15.5	1671	1.06	10.58	11.64	0.75	0.70	0.33	7.38	7.71	3.93	0.25
L.T.	2/25	14.8	1613	1.06	9.73	10.79	0.73	0.67	0.36	7.32	7.68	3.11	0.21
R.H.	2/25	18.1	1628	1.06	9.34	10.40	0.57	0.64	0.32	7.31	7.63	2.77	0.15
Diet C. High cereal with pasteurized milk													
A.C.[1]	3/27	18.4	1624	0.67	11.58	12.25	0.67	0.75	0.53	7.75	8.28	3.97	0.22
B.C.	3/27	13.7	1276	0.67	8.84	9.51	0.69	0.75	0.22	6.71	6.93	2.58	0.19
E.I.	3/27	23.1	1992	0.67	13.48	14.15	0.61	0.71	0.31	9.60	9.91	4.24	0.18
Diet D. High cereal with evaporated milk													
D.B.	4/7	17.0	1453	0.80	9.81	10.61	0.62	0.73	0.33	8.11	8.44	2.17	0.13
D.P.	4/7	16.4	1494	0.80	9.99	10.79	0.66	0.72	0.45	8.45	8.90	1.89	0.12
	Average	17.4	1614	0.77	10.12	10.89	0.63	0.68	0.35	7.46	7.81	3.08	0.18

[1] Due to a slight regurgitation on one day, these results are not included in averages.

lower ingestion level (0.40 mg. per kilogram). Perhaps the 50 per cent margin of safety advocated by these authors is not necessary; on the other hand, a depleted child may require considerably more iron than the estimated allowance. But, since in the present study the retentions obtained with diets containing 0.59, 0.65, or 0.75 mg. per kilogram were approximately the same, it would seem that a considerable excess of iron was contained in some of the diets used, and that 0.60 mg. per kilogram should meet the needs for maintenance and growth of the average child of the age studied.

Copper

The daily intake of copper of the children studied varied from 0.069 to 0.113 mg. per kilogram, or from 0.074 to 0.110 mg. per 100 Calories of food ingested. Since the amount of copper needed is not known, an attempt was made to estimate the needs by a study of the ratio of iron to copper in biological materials. As has been suggested, the iron retention may depend on the available copper, or vice versa. The diets fed in the study herein reported contained from seven to nine times as much iron as copper.

The copper excretion in the urine of the children studied was exceedingly small, the amount varying from 0.03 to 0.07 mg. per day. Similar small excretions are reported by Hess, Supplee, and Bellis ('23), who found that the amount excreted in the urine by infants was 0.02 mg. per day, by children from 2 to 3 years of age approximately 0.07 mg., and by adults from 0.09 to 0.12 mg. per day. Rabinowitch ('33), who analyzed urine from fifty adults, obtained values for urinary copper ranging from mere traces to 0.4 mg. per liter. In the present study, there was found to be no outstanding relationship between ingestion and urinary excretion. However, Hess et al. reported that adults on a high copper diet excreted more copper in the urine than on a low copper intake, and Rabinowitch found that urinary copper was increased by administering inorganic copper.

TABLE 4

Average daily intake, excretion, and retention of copper

NAME	DATE	WEIGHT	ENERGY INTAKE	COPPER INTAKE					COPPER EXCRETION			COPPER RETENTION	
				Milk	Food	Total	Per kg.	Per 100 Cals.	Urine	Feces	Total	Total	Per kg.
Diet A. Meat with pasteurized milk													
		Kg.	*Cals.*	*Mg.*	*Mg.*	*Mg.*	*Mg.*	*Mg.*	*Mg.*	*Mg.*	*Mg.*	*Mg.*	*Mg.*
A.C.	2/3	18.0	1677	0.12	1.11	1.23	0.069	0.074	0.03	0.90	0.93	0.30	0.016
B.C.	2/3	13.5	1272	0.12	0.83	0.95	0.070	0.075	0.03	0.76	0.79	0.16	0.012
E.I.	2/3	22.6	1974	0.12	1.46	1.58	0.070	0.080	0.04	1.01	1.05	0.53	0.023
L.T.	3/5	15.0	1613	0.17	1.21	1.38	0.093	0.086	0.04	0.62	0.66	0.72	0.048
R.H.	3/5	18.1	1628	0.17	1.20	1.37	0.076	0.084	0.04	0.77	0.81	0.56	0.031
Diet B. Meat with evaporated milk													
A.C.	2/11	18.3	1677	0.30	1.11	1.41	0.077	0.084	0.06	0.91	0.97	0.44	0.024
B.C.	2/11	13.7	1272	0.30	0.90	1.20	0.087	0.094	0.07	0.88	0.95	0.25	0.018
E.I.	2/11	23.0	1974	0.30	1.63	1.93	0.084	0.098	0.04	1.60	1.64	0.29	0.013
G.O.	2/25	15.5	1671	0.36	1.33	1.69	0.109	0.101	0.03	1.18	1.21	0.48	0.030
L.T.	2/25	14.8	1613	0.36	1.33	1.69	0.113	0.105	0.04	1.15	1.19	0.50	0.034
R.H.	2/25	18.1	1628	0.36	1.28	1.64	0.091	0.101	0.03	1.03	1.06	0.58	0.032
Diet C. High cereal with pasteurized milk													
A.C.[1]	3/27	18.4	1624	0.10	1.68	1.78	0.097	0.110	0.05	0.97	1.02	0.76	0.041
B.C.	3/27	13.7	1276	0.10	1.13	1.23	0.090	0.096	0.04	0.73	0.77	0.46	0.034
E.I.	3/27	23.1	1992	0.10	1.90	2.00	0.087	0.100	0.06	1.33	1.39	0.61	0.026
Diet D. High cereal with evaporated milk													
D.B.	4/7	17.0	1453	0.16	1.26	1.42	0.084	0.098	0.04	0.90	0.94	0.48	0.028
	4/7	16.4	1494	0.16	1.28	1.44	0.088	0.096	0.04	1.02	1.06	0.38	0.023
	Average	17.4	1614	0.21	1.27	1.48	0.086	0.091	0.04	0.99	1.03	0.45	0.026

[1] Due to a slight regurgitation on one day, these results are not included in averages.

By far the greater proportion of the copper was excreted in the feces, the amount varying from 93 to 98 per cent of the total excretion, values comparable to those obtained for the fecal iron.

The amount of copper retained by the children studied varied from 0.012 to 0.048 mg. per kilogram (average 0.026 mg.), the larger retentions in general being obtained with the diets containing the most copper. For example, B.C. (diet C) received 0.090 mg. and retained 0.034 mg. per kilogram, whereas with ingestions of 0.070 and 0.087 (diets A and B) she retained only 0.012 and 0.018 mg. respectively. Similarly, G. O., L.T., and R.H., with ingestions of 0.109, 0.113, and 0.091 mg. per kilogram (diet B) retained 0.030, 0.034, and 0.032 mg. per kilogram respectively. It would seem, therefore that there was no very large excess in these diets which were presumably high in copper. Indeed, some of the diets may have contained too little.

The amount of copper retained bore no constant relationship to the iron retained, the ratios of the iron-copper retentions varying from 4.7 to 15.8. This would indicate that some children were more depleted in copper, while others were either more depleted in iron or there was an insufficient amount of copper in the diets to meet the needs of the particular child. With the higher copper retentions, there was found to be a closer relationship between iron and copper (ratio 5:1) suggesting that for a given species, there may be a rather definite iron-copper retention ratio.

The highest copper retentions (0.034 and 0.048 mg. per kilogram) were obtained with minimum ingestions of 0.090 and 0.093 mg. per kilogram. Therefore, until further data are available, it is suggested tentatively that diets for children of the preschool age should include not less than 0.100 mg. of copper per kilogram of body weight.

SUMMARY

Fifteen iron and copper balance studies have been made with eight normal children ranging in age from 4 to 6 years. In a few cases successive studies were made with the same child.

The average iron retention for the group was found to be 0.18 mg. per kilogram, varying from 0.12 to 0.25 mg. with different children. High retentions, often found during first metabolism periods, were interpreted as being due to previous depletion. Diets containing 0.75 mg. per kilogram resulted in no higher retentions than those containing 0.59 or 0.65 mg. It is concluded, therefore, that 0.60 mg. per kilogram will meet the maintenance and growth needs for normal children of the ages studied.

The average retention of copper was found to be 0.026 mg. per kilogram at an average ingestion level of 0.086 mg. per kilogram. But since higher retentions (0.030 to 0.034 mg. per kilogram) were obtained at ingestion levels of 0.090 to 0.093, it is suggested that children of the ages studied should be given no less than 0.100 mg. per kilogram.

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THE INFLUENCE OF PREVIOUS DIET, GROWTH AND AGE UPON THE BASAL METABOLISM OF THE RAT

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(Received for publication November 27, 1933)

In addition to the previously reported findings obtained in our comprehensive metabolic study of the rat, considerable data have been collected dealing with the effects of previous diet, growth, age and sex upon the basal heat production of this animal. These data were secured with exactly the same type of rat, under the same laboratory and dietetic conditions (except where specially modified), and with the same metabolism technic as described in our preceding papers (Benedict, '30; Benedict, Horst and Mendel, '32; Horst, Mendel and Benedict, '30, '34 a, '34 b). In some of our experiments it was noted that when the diet of eight rats (about 100 days old) was changed from natural foods to a synthetic diet, that is, a mixture of purified food materials, the basal metabolism decreased. Observations with older rats were therefore made, to compare further the metabolic effects of natural and synthetic foods. Another phase of our study of the influence of diet upon metabolism dealt with the effects of different levels of protein in the diet, the maximum percentage being limited to an amount that would not produce hypertrophy of the kidneys (Osborne, Mendel, Park and Winternitz, '27). The differences in the rates of growth of our rats, dependent upon dietetic conditions, resulted in,

1) animals that may properly be designated as 'rapid growth' rats (average daily gain in weight about 4 gm.) and, 2) 'slow growth' rats (average daily gain in weight about 2 gm.). The basal metabolism of these animals was compared at different ages throughout the first year of life. In some young and half-grown rats, growth was deliberately retarded by feeding a 'normal' diet insufficient in quantity. Our measurements of the metabolism of these 'stunted' rats enable a comparison with the metabolism of normal rats of the same age but larger weights and of the same weight but younger ages. In some of the stunted rats the metabolism during 'resumed' growth on full rations was likewise studied. Finally, data were obtained with both male and female rats that contribute information regarding the influence of old age on the metabolism. This factor of old age has been too little studied, partly because animals, after they have reached the adult stage, are rarely maintained in the laboratory for the specific purpose of noting the influence of age. The increased interest in the physiology of old age, stimulated by Sherman's discovery that the average length of life of rats can be increased 10 per cent by dietetic devices (Sherman and Campbell, '28, '30), makes any contribution to this question of old age of great importance. Although the majority of our measurements were made on male rats, enough were made on females, particularly in studying the age factor, to contribute some information regarding the influence of sex.

Natural versus synthetic foods

The synthetic diet used was a mixture of purified food materials[1] supplemented daily by 300 mg. dry brewers' yeast and 150 mg. cod liver oil. The natural diet consisted of dog biscuit, milk food,[2] and fresh lettuce. Six male rats, littermates, were fed the synthetic diet for from 6 to 7 weeks (initial age 80 days). Their basal metabolism was measured twice during this dietary regimen, viz., in the fourth and the

[1] The 'medium-protein' diet described on page 143.

[2] Whole milk powder, 60 per cent; cornstarch, 12 per cent; lard, 28 per cent.

seventh weeks. The natural diet was next fed, and the metabolism was determined under basal conditions after the rats had been 2 and 4 weeks, respectively, on this regimen. In all instances the animals were measured at 30°C., had been 24 hours without food, and (as was customary in all of our observations on rats) had lived at 28°C. for 24 hours before the experiments. We were dealing, therefore, not with the immediate after-effect of food but with the problem as to whether the different diets caused differences in body com-

TABLE 1

Basal metabolism of rats receiving synthetic and natural foods. (Average values)

FOOD	NUMBER OF RATS STUDIED	AGE	WEIGHT	ACTIVITY	HEAT PRODUCTION PER SQUARE METER[1] PER 24 HOURS		
					Minimum	Maximum	Average
		days	*gm.*	*p.ct.*	*Cal.*	*Cal.*	*Cal.*
Synthetic	6	109	276	14	649	796	719
Synthetic	6	126	273	16	596	730	679
Natural	6	140	305	18	707	895[2]	789
Natural	6	154	327	27	755	952[2]	868
Synthetic	3	166	335	18	609	729	684
Synthetic	2	194	341	21	635	740	688
Natural	2	168	354	15	689	721	705
Natural	3	193	371	14	706	781	748

[1] $S = 9.1 \times w^{2/3}$

[2] Active more than 20 per cent of period of measurement.

position that were reflected in the metabolism. The average results of this study are recorded in the upper half of table 1.

If the slight difference in age is disregarded, it would appear from these data that the diet of natural foods resulted in an increased metabolism. Thus the minimum values when the rats were on the synthetic diet are lower than the minimum values when they were on a natural diet. The comparison of the maximum values is complicated by the relatively excessive activity of the rats when receiving natural foods. These rats were seldom altogether quiet, i.e., there were continual slight

movements, whereas the rats on the synthetic diet often showed periods of complete quiescence alternated with periods of great restlessness. At 140 days with natural foods, the maximum heat production, unaffected by great activity, was 801 Calories or considerably higher than the maximum heat production of 730 Calories at 126 days with the synthetic diet. At 154 days, during the period of natural foods, all of the results except the minimum value of 755 Calories denote metabolism with high activities. The average heat production was lower during the period of the synthetic diet than during the period of natural foods. The grand average for the two series with the synthetic diet was 699 Calories as compared with the grand average of 829 Calories with the natural diet. Supplementary evidence secured with twenty-four other male rats from 91 to 196 days old that had been given a diet of natural foods for at least 2 weeks showed a uniformly high basal metabolism averaging 778 Calories per square meter of body surface (average activity 13 per cent). Another group of twenty-five males of the same ages, subsisting upon a synthetic diet, gave low values averaging 720 Calories (average activity 11 per cent). Differences in activity did not account for the difference in metabolic levels, for even with the factor of excessive activity eliminated (as is the case in the average values of 778 and 720 Calories cited above), the metabolism of the rats that had been receiving the natural foods was 8 per cent higher than that of the rats subsisting upon the synthetic diet. The picture is complicated, however, by the fact that with the six rats used for the special study the body weights remained practically unchanged during the period of feeding with the synthetic diet whereas they increased during the period on natural foods.

When the six rats in the special study were 154 days old, three of them were again given a synthetic diet and the other three continued to live on natural foods. Again two metabolism observations were made on each rat, at least 3 weeks apart and 2 weeks after the dietary alteration. The results obtained are recorded in the lower half of table 1. Although

too few animals were studied in this series to permit drawing any definite conclusions with regard to whether there is any sustained after-effect of a continued diet of natural foods, it would appear that, in general, the previous diet did not play a significant role in the metabolism of these rats.

The protein factor

In man and dogs the specific dynamic action of protein is higher than that of any other foodstuff. The pronounced immediate after-effect of protein has led to the general belief that rations rich in protein result in a high basal metabolism.

TABLE 2

Composition of diets

COMPONENTS	HIGH PROTEIN	MEDIUM PROTEIN	LOW PROTEIN
	p.ct.[1]	*p.ct.*[1]	*p.ct.*[1]
Casein[2]	60	30	6
Cornstarch	12	42	65.7
Butter fat	9	9	9
Lard	15	15	15
Salt mixture[3]	4	4	4
Cystine	0	0	0.3

[1] By weight.
[2] On an air-dry basis, the casein contained 13.3 per cent nitrogen.
[3] Described by Osborne and Mendel ('19).

Wang ('30) and her associates did not find any essential difference in the basal metabolism of normal women with protein intakes varying from 0.6 to 2 gm. per kilogram of body weight. The data of Hogan and Pilcher ('30) do not show any significant differences in the metabolism of rats with diets containing 12 and 24 per cent of casein. In our investigations sixteen male rats were used to study the effects of high-, medium- and low-protein diets, there being five rats in each of the first two groups and six in the low-protein group. These rats were from 90 to 120 days old at the inception of the protein regimen. Littermates were chosen in groups of three, one for each diet. The diets (table 2) were

so compounded that, although the quantity of casein varied, the total energy value remained 5.2 Calories per gram. Each rat received daily 300 mg. dry brewers' yeast and 145 mg. cod liver oil. The basal diets were fed ad libitum. The daily nitrogen consumption, calculated per 200 gm. of rat, averaged 494, 262 and 54 mg. in the high-, medium- and low-protein groups, respectively. Thus the rats in the low-protein group received less nitrogen than the minimum requirement for rats of 200 to 350 gm. observed by Osborne and Mendel ('15). Strict comparisons are not possible, however. In the older Osborne and Mendel experiments the nitrogen content of the protein-free milk was disregarded. On the other hand, the supplementary cystine was not used in their diets.

TABLE 3

Basal metabolism of rats on different protein levels

PROTEIN LEVEL	NUMBER OF RATS STUDIED	AGE		WEIGHT		HEAT PRODUCTION PER SQUARE METER PER 24 HOURS		
		Initial	Final	Initial	Final	Minimum	Maximum	Average
		days	*days*	*gm.*	*gm.*	*Cal.*	*Cal.*	*Cal.*
High	5	116	140	261	285	686	773	724
Medium	5	119	145	252	271	685	766	729
Low	6	118	145	249	247	639	719	677

The basal metabolism of each rat was determined at three different times, once when the rats had been on the special protein diets for about 2 weeks, and twice thereafter at intervals of about 2 weeks. The values observed in the three determinations on each rat were fairly constant. The results, recorded as the average heat production and the range observed for the rats in each dietary group, are shown in table 3. The initial ages and weights are those noted at the time of the first metabolism measurement, and not at the start of the feeding on the special diets. The values for the average metabolism and for the range show that there was practically no difference in the metabolism of the rats in the high- and medium-protein groups, but a definitely lower metabolism in the animals in the low-protein group. The rats in the latter

group likewise had a somewhat lower final weight. From the post-mortem examinations and from this finding of a low metabolism with the rats on the low-protein diet it can be concluded that deleterious physiological effects were beginning to appear in the rats on the extremely low protein diet.

Suppression of growth or stunting

In studies of the laws governing the growth of the rat, various methods (so-called 'stunting processes') of maintaining the body weight of the youthful rat at a constant level have been developed. The fact that during this stunting process the animal retains approximately its normal configuration without appearance of extreme emaciation removes the problem of the metabolic effect of suppression of growth completely from the well-known problem of undernutrition. In studies on undernutrition adult animals and humans have been made to lose a considerable proportion of their previously acquired body weight. In our investigation, on the contrary, the growth of the rat was suppressed by feeding insufficient quantities of a normal diet.[3] In one series, four male rats (about 31 days old at the start) received limited quantities of food for 42 days. Metabolism was measured four times, initially on the tenth day of experimental feeding and three times thereafter at intervals of about 10 days. The results are recorded in table 4, from which it can be seen that for 31 days the body weights of these rats remained almost stationary. The heat production also remained essentially constant, although with all four animals it was somewhat lower at the end of the series than at the start. The activity varied somewhat, but for the most part permitted comparison of the various values.

On the average the metabolism decreased from 708 Calories per square meter of body surface on the tenth day of stunting to 599 Calories on the thirty-first and the thirty-fourth days of stunting—a decrease of 15 per cent. During these

[3] The diets used in our experiments were similar to the 'low calorie' diets described by Winters, Smith and Mendel ('27).

24 days the rats had increased in age from about 40 to 65 days. At corresponding ages, the metabolism of seven normal male rats averaged 868 Calories (at 42 to 45 days) and 828 Calories (at 56 to 70 days) per square meter of body surface (table 8, p. 152). Hence, the heat production of the stunted

TABLE 4

Basal metabolism of male rats stunted by restriction of Calorie intake. (Measured at 30°C. and 18 to 24 hours after food)

RAT NO.[1]	AGE	WEIGHT	DAYS OF STUNTING	ACTIVITY	HEAT PRODUCTION PER 24 HOURS	
					Per 200 gm.	Per square meter
	days	*gm.*		*p.ct.*	*Cal.*	*Cal.*
1	45	56	10	5	33.6	707
	66	62	31	10	28.1	608
	73	63	38	6	30.8	674
	76	67	41	..	28.7	640
2	41	61	10	15	31.5	681
	50	64	19	7	29.7	651
	62	65	31	7	24.3	537
	72	67	41	10	28.7	640
3	41	57	10	15	33.3	704
	62	63	31	8	28.9	632
	65	65	34	13	27.7	612
	72	67	41	6	29.0	647
4	44	62	13	11	34.1	739
	62	63	31	19	30.5	667
	65	65	34	25	28.9	639
	72	67	41	17	30.7	687

[1] In this report and in our preceding reports the rats have been numbered consecutively, beginning with no. 1, for purposes of ready reference. The numbering has been done independently in each report, however, and rat 1 or rat 24, for example, in this article is not the same as rat 1 or rat 24 in our earlier papers.

rats was 18 per cent below normal on the tenth day of stunting (41 to 45 days old) and 28 per cent lower than normal on the thirty-first and thirty-fourth days of reduced food consumption. On the forty-first day of stunting the heat production averaged 654 Calories. At this time the stunted rats were 72 to 76 days old and weighed 67 gm. each. Twelve normal

males of about the same age but not of the same weight had an average basal heat production of 785 Calories per square meter of body surface (table 8, p. 152). Thus, on the basis of equal age, the average metabolism of the four stunted rats was 17 per cent below that of the normal rats. The metabolism of five normal males of about the same weight (69 gm.) as the 72-day-old stunted rats but much younger was, on the average, 834 Calories (unpublished data). Thus the metabolism of the stunted rats was 22 per cent below that of normal rats of the same weight.

In a second series of observations the basal metabolism of two male rats (nos. 5 and 6, table 5) was studied during 84 to 89 days of stunting. The metabolism decreased during the first 28 days, remained at about the same level for the next 7 days, and increased as the stunting period was prolonged. When these rats were 52 and 64 days old, respectively, and had been 28 days on the restricted diet, their heat production averaged 598 Calories or practically the same as the average of 599 Calories noted with the four rats in the first series with 31 to 34 days of reduced rations. On the eighty-fourth and eighty-ninth days of stunting the metabolism was 572 and 693 Calories, respectively. The greater activity of rat 6 at this time undoubtedly explains the difference observed in the metabolism of the two rats. The average metabolism of seven normal males from 101 to 126 days old or approximately the same age as these two stunted rats at the close of the period of reduced food intake was 761 Calories per square meter of body surface (table 8, p. 152). In all these studies there were at times unavoidable differences in activity. Indeed, the stunted animals, as seen from table 5, were unusually active during measurements. Nevertheless, the heat production was unquestionably lower in the stunted rats, in spite of their great activity, than in normal rats of the same age and likewise lower than in normal rats of the same weight.

At the end of the experiments the four stunted rats in the first series (table 4) were killed and their bodies analyzed for fat. The bodies of two normal rats of the same age but

weighing 128 and 137 gm., were likewise analyzed. The total fat in the stunted rats was 5.5, 1.1, 1.6 and 0.7 per cent of the total body weight as compared with 5.5 per cent in the two normal rats. The subcutaneous fat of three of the stunted rats averaged 0.7 per cent of the total body weight (for rat 1

TABLE 5

Basal metabolism of male rats stunted by restriction of Calorie intake. (Two rats in one chamber, measured at 28°C. and 24 hours after food)

RAT NO.	AGE	WEIGHT	DAYS OF STUNTING	ACTIVITY	HEAT PRODUCTION PER 24 HOURS	
					Per 200 gm.	Per square meter
	days	*gm.*		*p.ct.*	*Cal.*	*Cal.*
5	40	55	4	..	35.0	731
6	28	55				
5	44	60	8	37[1]	32.3	681
6	32	54				
5	51	58	15	32[1]	30.7	654
6	39	56				
5	55	56	19	31[1]	30.2	643
6	43	58				
5	64	60	28	21[1]	28.0	598
6	52	58				
5	71	58	35	19[1]	28.9	618
6	59	58				
5	82	60	46	35[1]	31.3	671
6	70	60				
5	89	60	53	46[1]	30.7	657
6	77	60				
5	97	59	61	60[1]	34.8	741
6	85	58				
5	107	61	71	16	31.5	681
	114	63	78	8	28.9	632
	120	68	84	10	25.6	572
6	98	61	74	40	32.8	709
	106	65	82	14	33.8	748
	113	69	89	18	30.7	693

[1] On the basis of the total activity of the two rats.

it amounted to 3.8 per cent). The subcutaneous fat of the normal rats amounted to 3 per cent. Thus the normal rats were better protected against low external temperatures than the stunted rats.[4]

Resumed growth after stunting

It is a known fact that rats whose growth has been retarded for some time will gain regularly in body weight with resumed liberal feeding. With such rats it is possible to study the metabolism during growth at an age when growth has normally ceased. Five male rats (114 to 135 days old) that had been living on inadequate rations (deficient in various essential factors) for from 73 to 98 days were subsequently given a diet of natural foods. The metabolism was measured at the end of the period of retarded growth and on three different occasions during the period of resumed growth, as shown in table 6. In all determinations the activity was less than 15 per cent. The heat production at the end of the period on inadequate diets averaged 706 Calories per square meter of body surface, or somewhat lower than the value, 761 Calories (table 8, p. 152), for seven normal male rats of about the same age. The metabolism apparently did not increase immediately after the diet was changed, for the heat production of rat 7 per unit of weight and of body surface was somewhat lower 3 days after realimentation than it was during the period of suppressed growth. On the seventh to the tenth days of resumed growth, the heat production per square meter of body surface was 9, 11 and 14 per cent higher than the metabolism with inadequate diets (rats 8 to 10). By the eighteenth day of realimentation, the metabolism per unit of surface area had reached a maximum, being 33 and 15 per cent above the level at the close of the stunting period (rats 7 and 8). Thereafter, the metabolism per unit of surface area gradually decreased, varying somewhat with the individual rat. In the case of rats 7 and 9 the experiments were con-

[4] We are indebted to Dr. L. L. Reed of Yale University for the fat determinations.

tinued for 75 and 77 days. At the close of the study these rats weighed 406 and 354 gm., respectively, weights not much different from the average body weights of 429 gm. (table 7, p. 152 and 341 gm. (table 8, p. 152) for normal rats of about the same age. Likewise, the heat production of rats 7 and 9, 707 and 664 Calories, was not very far from the average metabolism of normal rats of the same age, 755 Calories being observed on eleven rapid growth rats (table 7) and 714 Calories on eight slow growth rats (table 8). It would thus appear that with realimentation the stunted rat not only may resume its normal growth unhampered but that its metabolic level when normal growth is attained will be not far from that of the normally growing rat of the same age and weight.

TABLE 6

Basal metabolism of male rats in relation to resumption of growth after retardation. (Measured at 30°C. and 24 hours after food)

RAT NO.	PERIOD OF GROWTH	AGE	WEIGHT	DAYS OF RESUMED GROWTH	HEAT PRODUCTION PER 24 HOURS	
					Per 200 gm.	Per square meter
		days	*gm.*		*Cal.*	*Cal.*
7	Retarded	118	129	..	23.7	670
	Resumed	121	142	3	22.1	633
		135	239	17	26.2	892
		170	345	52	20.5	788
		193	406	75	17.4	707
8	Retarded	119	71	..	32.9	750
	Resumed	126	107	7	31.2	815
		137	143	18	30.1	863
		161	230	42	25.0	842
9	Retarded	114	109	..	25.4	663
	Resumed	121	150	7	26.0	759
		167	305	53	18.7	692
		191	354	77	17.1	664
10	Retarded	135	119	..	27.5	741
	Resumed	145	172	10	26.8	822
		166	229	31	24.2	812
11	Resumed	112	118	16	31.4	845
		161	273	65	23.8	846

Even after stunting, therefore, rats may be made to grow and reach a size approximately normal for albino rats of similar strains. As can be seen from table 6, these rats had a remarkably rapid growth after liberal feeding, the most striking case being that of rat 7, which between the ages of 118 and 135 days, that is, during 17 days of realimentation, increased in weight from 129 to 239 gm. or at the rate of more than 6 gm. per day. Hence it is clear that the problem of resumed growth and metabolism is closely bound up with the question of rate of growth.

Rate of growth

The usual rate of growth of the normal male rat between the ages of 30 and 90 days is about 2.3 gm. per day (Donaldson, '24), but with special diets much more rapid rates of growth are readily secured. Our experiments included observations on a group of rats designedly fed so that they gained weight at the rate of 4 gm. or more per day between the ages of 30 and 90 days. These were our so-called 'rapid growth' rats (table 7). These rats received a synthetic food mixture[5] with daily supplements of 20 gm. of fresh lettuce and 200 to 400 mg. dry brewers' yeast. Another group of rats designated 'slow growth' (table 8) were fed our standard synthetic diet[6] with quantities of yeast limited to obtain the desired gain of about 2 gm. a day. Initially, 100 to 200 mg. of yeast were fed daily. This was gradually increased to 300 and 400 mg. In comparing these two groups of rats, we have to deal with the fact that the rapid growth rats were much heavier than the slow growth rats at the same ages, and again we are confronted by the problem as to the best physiological method of comparing the metabolism of animals of different sizes. If nothing more than for the purpose of showing the extreme complexity of a problem that has hitherto been assumed very simple, we have retained the body-surface comparison in the heat values recorded in tables 7 and 8. In all

[5] Described by Osborne and Mendel ('26).
[6] Described by Horst, Mendel and Benedict ('34 a).

instances the rats were active for less than 18 per cent of the period of measurement and usually less than 10 per cent.

TABLE 7

Basal metabolism of 'rapid growth' rats. (Males that gained 4 gm. daily between the ages of 30 and 90 days; measured at 30°C. and 24 hours after food)

NUMBER OF RATS	AGE RANGE	WEIGHT		AVERAGE ACTIVITY	HEAT PRODUCTION PER 24 HOURS		
					Average per 200 gm.	Per square meter	
		Range	Average			Range	Average
	days	*gm.*	*gm.*	*p.ct.*	*Cal.*	*Cal.*	*Cal.*
14	42– 55	94–139	123	10	33.8	766–1041	923
13	56– 69	122–198	154	11	28.9	776– 997	849
11	71– 85	196–321	230	10	24.2	752– 891	810
5	88– 99	246–275	262	11	23.8	787– 895	836
6	101–130	283–348	321	11	19.3	676– 799	725
10	136–159	332–500	393	11	18.6	653– 882	745
11	162–224	354–487	429	10	18.2	643– 854	755
14	233–294	433–664	521	8	15.9	620– 800	701

TABLE 8

Basal metabolism of 'slow growth' rats. (Males that gained 2 gm. daily between the ages of 30 and 90 days; measured at 30°C. and 24 hours after food)

NUMBER OF RATS	AGE RANGE	WEIGHT		AVERAGE ACTIVITY	HEAT PRODUCTION PER 24 HOURS		
					Average per 200 gm.	Per square meter	
		Range	Average			Range	Average
	days	*gm.*	*gm.*	*p.ct.*	*Cal.*	*Cal.*	*Cal.*
7	42– 45	70– 98	83	7	36.5	798–948	868
11	56– 70	100–138	119	8	30.6	727–909	828
12	71– 85	120–171	140	9	27.6	701–845	785
14	87– 99	150–188	175	11	25.8	708–952	792
7	101–126	166–242	218	8	23.2	611–815	761
17	131–148	224–281	248	10	21.3	646–851	734
8	170–226	312–364	341	9	18.6	587–833	714
13	232–302	307–402	359	11	18.8	687–818	734

Fourteen 'rapid growth' rats at 42 to 55 days of age and weighing from 94 to 139 gm. had an average heat production of 33.8 Calories per 200 gm. of body weight and 923 Calories per square meter of body surface. Seven 'slow growth' rats

from 42 to 45 days of age but weighing 40 gm. less on the average than the rapid growth rats showed average heat values per unit of weight and body surface of 36.5 and 868 Calories, respectively. Here the picture is exactly the opposite on the two bases of comparison, namely, the slow growth rats had a lower heat production than the rapid growth rats per unit of surface area and a higher heat production per unit of weight. The heat values per 200 gm. of weight are in full conformity, however, with the general finding that animals of the same species usually have a larger heat production per unit of weight, the smaller the animal.

At 56 to 69 days of age thirteen rapid growth rats had an average heat production of 849 Calories per square meter of body surface, whereas eleven slow growth rats of the same age had an average metabolism of 828 Calories. Thus, on this basis, the slow growth rats had a slightly lower metabolism than the rapid growth rats. Per unit of weight, however, the slow growth rats had a higher heat production than the rapid growth rats, the average values being 30.6 and 28.9 Calories, respectively. At these ages the slow growth rats weighed 35 gm. less, on the average, than the rapid growth rats. At 71 to 85 days of age eleven rapid growth rats had an average metabolism of 810 Calories and twelve slow growth rats of the same age, 785 Calories. The body weights of the slow growth animals were 90 gm. less, on the average, than those of the rapid growth animals, and the heat production per 200 gm. of weight was higher, 27.6 as compared with 24.2 Calories.

From 88 to 99 days of age, the metabolism of five rapid growth rats averaged 836 Calories per square meter of body surface; that of fourteen slow growth rats, 792 Calories. Hence, on the body surface basis, the metabolism of the rapid growth rats was still definitely higher than that of the slow growth rats. Per unit of weight, however, the average metabolism of the rapid growth rats, 23.8 Calories, was lower than the average heat production of the slow growth rats, 25.8 Calories. At these ages, the slow growth rats weighed 87 gm. less, on the average, than the rapid growth rats.

At 88 to 99 days the metabolism (per unit of surface area) of both the rapid growth and the slow growth rats was somewhat higher than that at 71 to 85 days of age. As the average age of sexual maturity of male rats has been found to be 109 days (Slonaker and Card, '23), the higher metabolism of our rats at 88 to 99 days suggests a prepubertal rise similar to that observed in boys by DuBois ('16) and recently confirmed by Topper and Mulier ('32) and Bruen ('33). The Nutrition Laboratory did not find this rise with girls (Benedict and Hendry, '21).

In general, comparisons on the body surface basis show that the metabolism of the rapid growth rats from 40 to 100 days of age (the period during which growth proceeded at the rate of 4 gm. a day) was higher than that of the slow growth rats. From 101 to 226 days of age, however, the metabolism of the two groups did not differ appreciably. From 233 to 294 days of age, the rapid growth rats had a slightly lower metabolism than the slow growth animals. At this age the rapid growth rats weighed from 433 to 664 gm. and the slow growth rats from 307 to 402 gm.

The most striking metabolic differences between the two groups of rats appear in the early stages of life, when the factors of rate of growth and age are most prominent. The picture of higher metabolism in the rapid growth rats per square meter of body surface is offset in large part by the finding that per 200 gm. of body weight their metabolism is the same or slightly lower than that of the slow growth rats. In the light of the comparisons at the older ages it is probably reasonable to conclude that the rate of growth is practically without effect upon the basal metabolism.

Metabolism in old age

In addition to our observations on the metabolism during the period of growth, when there are simultaneously changes in body weight and in age, we had occasion to study the metabolism of a few male and female rats in old age. These

animals[7] received a stock diet of dog biscuit and wheat germ food[8] which they ate ad libitum. The adjuvants, fresh lettuce or cabbage, were fed twice each week.

Six of our male rats (nos. 12 to 17, table 9) were allowed to live far beyond the usual time allotted to rats in laboratory studies. Four of these showed increases in weight during the period of observation and two showed no increases. The important feature of the study, however, is that the metabolism measurements on these animals were continued into the third year of life. Although there were considerable fluctuations in the metabolism of the rats recorded in table 9, especially in the last months, in general one can conclude that the metabolism of the male rat remains at a practically constant level during the second and third years of life. This conclusion is based upon experiments with a small number of rats, and further data are needed. Moreover, the situation is somewhat complicated by the fact that these rats were for the most part unusually large. Three certainly were extremely large, and the other three were well over the average weight for the normal adult male rat. It is unfortunate that in the study of this particular problem the same rat was not observed over a longer period of time, although the measurements on two of these animals (nos. 12 and 17) did extend over nearly a year.

Fourteen female rats (nos. 18 to 31) were studied during the second and third years of life. The ages of rats 28 to 31 ranged from about 300 or 450 to 800 or 900 days. The weights of nine of the rats (nos. 18 to 26) ranged from 218 to 299 gm. Five of the rats (nos. 27 to 31) weighed over 300 gm. In fact, rat 30 weighed nearly 500 gm. toward the end of the period of study—an extremely heavy weight for a female rat. The heat production per square meter of body surface (table 10) was in the majority of instances materially

[7] Rats 15 and 16 were 'rapid growth' rats and received the diet explained on page 151 until they were about 870 days of age. Thereafter, they were fed the stock diet.

[8] Whole milk powder 50 per cent, wheat germ 20 per cent, lard 30 per cent.

under 670 Calories, indeed often under 600 Calories. If allowances are made for differences in activity (allowances

TABLE 9

Basal metabolism of male rats in the second and third years of life. (Measured at 28° to 30°C. and 24 hours after food)

RAT NO.	AGE	WEIGHT	RECTAL TEMPERATURE	ACTIVITY	HEAT PRODUCTION PER 24 HOURS	
					Per 200 gm.	Per square meter
	days	*gm.*	*°C.*	*p.ct.*	*Cal.*	*Cal.*
12	393	517	37.4	..	16.2	713
	459	553	37.0	..	15.1	681
	566	554	37.8	..	12.8	576
	601	543	37.8	..	16.5	737
	646	545	38.1	6	14.0	628
	709	504	....	..	14.7	643
13	467	567	37.2	7	16.5	750
	514	591	37.1	5	14.9	686
	645	602	37.2	..	14.8	687
	659	594	37.8	..	15.5	717
	746	660	37.3	..	16.3	781
	774	600	38.2	..	17.9	831
14	764	345	37.8	12	17.8	688
	780	348	38.2	23	18.2	702
	883	321	37.6	4	20.0	749
15	807	405	36.4	21	19.1	777
	828	414	35.6	..	17.4	711
	835	416	37.2	..	17.5	720
	889	430	35.9	..	18.7	776
	896	429	36.0	23	20.3	840
16	804	344	37.3	21	19.0	729
	832	331	36.6	3	18.1	690
	840	343	36.8	13	16.3	628
	879	348	35.6	..	19.8	767
	889	374	35.9	8	17.5	695
	969	447	37.4	14	20.0	840
	1060	400	36.2	7	18.1	735
17	443	500	36.7	8	16.1	702
	513	546	37.1	..	14.1	632
	649	540	37.8	..	15.5	694
	684	558	37.6	13	13.6	615
	759	547	38.0	..	14.2	639

made only with difficulty), it is evident that, in general, the metabolism of these female rats was not materially altered during the second and third years of life. Indeed, compared with females at ages between 62 and 200 days, the metabolism was lower during the second and third years of life. These results accord with the results obtained on the male rats (table 9), although the metabolism of the males per unit of surface area was somewhat higher, on the whole, than that of the females of similar ages.

The rectal temperatures of these male and female rats, obtained at the time of the metabolism measurements, are also recorded in tables 9 and 10. Although we recognize that there are not enough temperature records to permit making any deductions, we have included the data in the tables simply as a general contribution regarding the physiology of old age.

The age factor with female rats was of unusual interest to us, in view of the finding of Benedict and MacLeod ('29) that, contrary to practically all animals thus far studied by the Nutrition Laboratory, female rats of the colony raised at Columbia University had unquestionably an increasing metabolism per unit of surface area with advancing age. This trend is so clearly shown by the chart published by these investigators that there is little doubt as to its probability. Male rats, much fewer in number, studied at that same time, gave by no means the same clear picture of increasing metabolism with advancing age, but certainly showed no tendency for a decrease in metabolism with age. The results with the Yale female rats are entirely the opposite of those noted with the Columbia female rats. Careful analysis of the data indicates that after about the first year the weight of the Columbia rats remained fairly constant, whereas that of the Yale rats continued to increase, certainly until they were at least 500 days old. Indeed, the Yale rats of both sexes weighed, on the average, from 70 to 75 per cent more, in the second and third years of life, than did the Columbia rats. Combined with the factor of increasing age, therefore, there are the factors of continually increasing body weight and of markedly

TABLE 10

Basal metabolism of female rats in the second and third years of life.[1] *(Measured at 30°C. and 24 hours after food)*

RAT NO.	AGE	WEIGHT	RECTAL TEMPERATURE	ACTIVITY	HEAT PRODUCTION PER 24 HOURS		RAT NO.	AGE	WEIGHT	RECTAL TEMPERATURE	ACTIVITY	HEAT PRODUCTION PER 24 HOURS	
					Per 200 gm.	Per sq.m.						Per 200 gm.	Per sq.m.
	days	*gm.*	*°C.*	*p.ct.*	*Cal.*	*Cal.*		*days*	*gm.*	*°C.*	*p.ct.*	*Cal.*	*Cal.*
18	436	299	36.9	12	19.6	722	28	274	235	38.7	8	20.2	686
	455	297	38.0	3	18.2	667		288	238	37.4	..	22.7	774
								440	282	37.0	8	20.7	746
19	494	249	35.9	..	18.2	631		494	284	38.7	10	18.0	651
	517	250	35.1	..	19.0	657		531	297	39.1	9	16.3	597
								595	308	38.7	2	17.0	632
20	493	270	37.2	2	17.3	616		658	314	38.3	5	16.6	620
	496	271	37.3	12	17.3	617		721	315	38.1	9	18.4	688
	573	288	37.8	5	19.2	698		763	330	38.4	6	17.2	652
	580	293	37.2	4	17.6	643		838	324	37.6	..	17.9	675
21	493	239	36.7	11	19.5	666	29	454	344	38.6	3	14.7	565
	496	238	37.3	8	18.0	613		503	336	38.7	14	15.6	597
	573	244	37.6	4	20.1	690		551	396	38.3	6	15.7	633
	580	250	36.3	6	18.6	643		604	385	39.0	4	13.2	528
								650	389	38.2	5	13.9	557
22	516	288	37.2	5	16.3	589		730	374	37.3	12	16.2	643
	525	285	37.4	7	15.4	558		792	363	37.8	..	17.2	675
								867	356	36.7	..	16.2	631
23	559	218	37.8	8	23.4	773							
							30	453	334	37.9	2	16.8	642
24	530	234	....	..	21.2	717		502	347	37.3	11	15.0	579
	550	242	36.4	9	21.0	720		628	382	38.8	9	16.0	637
								656	410	38.3	13	16.3	664
25	550	293	36.4	..	16.8	613		726	484	38.3	..	14.3	617
	573	271	35.7	..	18.4	654		757	488	38.4	21	14.9	643
								832	480	37.7	27	15.7	674
26	567	267	36.2	..	20.4	724		856	458	38.6	18	15.5	658
	590	263	35.8	..	23.3	821		895	448	36.7	4	16.1	678
27	536	310	37.6	8	17.0	628	31	580	375	37.2	6	15.9	632
	547	323	38.4	6	16.4	617		622	362	38.3	20	17.7	692
	733	339	37.4	24	19.0	714		674	373	38.1	9	15.9	631
	742	331	38.1	12	17.0	646		627	382	37.8	16	14.4	573
								758	375	38.3	..	16.3	646
								807	376	37.8	8	15.0	593
								856	373	37.2	16	17.0	675
								888	386	37.0	14	17.2	687
								973	397	37.8	..	18.3	738

[1] Previously mated but studied when not pregnant and not lactating.

different body weights for age with the two groups. Thus in this comparison we encounter again the as yet wholly unsolved problem as to how the heat values can be expressed to rule out differences in size. It is clear that calculations per unit of body weight do not eliminate this factor, and it is becoming increasingly evident that there is no correction for this factor in the calculations per unit of surface area. With unusually large rats we found that the heat production per square meter of surface area was smaller, the larger the rat (Benedict, Horst and Mendel, '32). Comparison of the heat values on this same basis for the Columbia and Yale female rats substantiates this finding, the lighter weight Columbia rats having a higher metabolism per unit of surface area in the second and third years of life than the heavier Yale rats. If there were any method of calculating the heat values to correct for differences in size (and thus far we have been unable to find any satisfactory substitute for the traditional calculations per unit of weight and surface area), it is conceivable that the heat production of the Columbia rats,[9] instead of increasing with advancing age, might show the same tendency as did that of the Yale rats to remain unaltered at the older ages.

SUMMARY

The basal metabolism of male rats (91 to 196 days old) that received a diet of natural foods was somewhat higher initially (2 weeks after the diet was changed) than the metabolism of other male rats on a so-called synthetic diet. When these diets were fed to rats for a continued length of time, however, they played no significant role in the metabolism.

There was practically no difference in the basal metabolism of rats fed diets of high-protein and medium-protein content,

[9] These earlier results were obtained before the importance was recognized of keeping the animals at 28°C. for 24 hours before the metabolism measurements. In the few instances where female rats of the Columbia series were measured at 28° after living for 24 hours at this same temperature, it was noted that the metabolism did not increase with old age. Further experiments on this particular problem are at present in progress with the Columbia series of rats.

but rats on a low-protein diet had a lower metabolism and in these rats post-mortem examinations showed that the diet had begun to have a deleterious physiological effect.

The basal heat production of young rats stunted in growth was lower than that of normal rats of the same age but larger weight and lower than that of normal rats of the same weight but different age. With realimentation the stunted rat not only resumed its normal growth but its basal metabolism, when normal growth was attained, was at approximately the same level as that of the normally growing rat of the same age and weight.

The rate of growth, whether slow or rapid, did not have any appreciable effect upon the basal metabolism of normal male rats. However, from 40 to 100 days of age the heat production per unit of surface area of the rapidly growing rats was somewhat higher than the metabolism of the slowly growing rats, whereas at 230 to 300 days of age (weight 433 to 664 gm.) the metabolism of the rapid growth rats was slightly lower than that of the slow growth rats (weight 307 to 402 gm.).

Male rats in the second and third years of life had a higher metabolism than females of the same age.

The basal metabolism of both male and female rats remained at practically a constant level during the second and third years of life. This finding is not in accord with an earlier observation on female rats from a different rat colony, that the heat production increased with increase in age between 2 and 28 months. Analysis of both sets of data indicates a very great difference in the body weights of the two groups of rats. The lack of agreement in the findings may be explained by the supposition that this difference in body size is not ruled out either in the expression of the heat production per unit of weight or that per unit of surface area.

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STUDIES ON THE EFFECT OF HIGH DOSES OF IRRADIATED AND NON-IRRADIATED ERGOSTEROL ON THE ALBINO RAT [1]

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(Received for publication November 2, 1933)

The very earliest studies with irradiated products indicated that they had a toxic action, and likewise the exposure of the animal to light also indicated toxic action. However, later studies led to irregular reports by various investigators. These irregularities were in part due to the fact that some of the investigators reported their dosages in terms of milligrams of ergosterol and not in terms of the therapeutic dose and also because the methods of irradiation used at that time undoubtedly introduced variable potencies and toxicity.

It is not our purpose to discuss all the papers on this subject, but to emphasize only the most important studies on rats. Thus the work of Rosenheim and Webster ('27) showed that 10,000 times the minimum antirachitic dose was not lethal to rats, while the work of Pfannenstiel ('28) and of Kreitmar and Moll ('28), using 'enormous doses,' claimed pathhological findings for several species and also a species variation. Dixon and Hoyle ('28) fed a moderate excess of irradiated ergosterol to rats on a normal bread and milk diet and found no appreciable effect. Similar rats on a synthetic basal diet fed five and eight times larger doses failed to gain weight and on autopsy showed calcium phosphate in the urinary

[1] This work was undertaken on the suggestion of Mead Johnson & Co. who supplied the funds for the first year and who throughout the entire period have supplied us with the ergosterol preparations.

tract. Subsequently ('29) they fed even larger doses and substantiated the above findings. About the same time, Harris and Moore ('28) reported that on a synthetic diet, a dose 1000 times the minimum antirachitic dose was harmless to rats, while 100,000 times overdosage was rapidly fatal. The material used was ergosterol irradiated in an oily medium. They interpret their results to indicate that the irradiated ergosterol itself and not unchanged ergosterol or decomposition products was the factor responsible for the toxic action. These workers claimed to have obtained all the pathological findings reported by the earlier workers. Hoyle ('30), with rats on both synthetic and ordinary bread and milk diets, showed that when a synthetic diet was used he could readily obtain all the striking results of the earlier workers and also confirmed the findings of Harris and Moore. He further showed that if he fed high enough concentrations of irradiated ergosterol on a bread and milk diet he then could also obtain some toxic results although they were not as striking as on a synthetic diet. However, he showed that neither bread alone nor milk alone could prevent this condition, but that the two together acted as a preventative, and therefore concluded that there are two factors which one has to consider in the production of this condition, namely: a) a synthetic diet and, b) an excess of irradiated ergosterol. And finally, he showed that the toxic action was due to a) an excess of irradiated ergosterol and, b) an unknown toxic substance which was much more readily produced when the ergosterol was irradiated in an alcohol solution, being only difficultly produced when the irradiation was carried on in oil. Holtz and Schreiber ('30), working on mice, rabbits and dogs, confirmed the contention of Hoyle and showed that the antirachitic factor and the toxic factor are two separate principles. Furthermore, they showed that the antirachitic activity does not parallel the toxic action in all preparations. Seel ('29) showed that in rabbits large doses of irradiated ergosterol caused a marked loss of weight, emaciation and other cachectic symptoms, and that animals which were rachitic were more

sensitive to larger doses than were normally fed animals. Vara-Lopez ('30) showed that large doses of Vigantol caused changes in the spleen, adrenals and entire reticulo-endothelial system which were peculiar to the Vigantol solution not being given by Vigantol tablets, cod liver oil, or by cholesterol in oil. Goebel ('32) demonstrated that preparations of irradiated ergosterol differing as to industrial source or duration of activation differ also in their toxic effect when fed in massive doses. Bills and Wirick ('30) report experiments running over a longer period of time than the previous work, and showed that 1000 times the therapeutic dose caused just perceptible effects, 4000 times the therapeutic dose was definitely injurious, and 40,000 times the therapeutic dose was strongly toxic. In their work they used both the McCollum 3143 rachitic diet and the stock diet which is used in that laboratory and which cannot be considered as a bread and milk diet, according to Hoyle and his co-workers.

Our work was started in December, 1928. It was our object to maintain throughout our experimental procedures as normal conditions as possible and to limit our observations to only one commercial product which was prepared and furnished by Mead Johnson & Co.

EXPERIMENTAL METHODS

A. Method of assay employed

1. Line test. The method of assay used was to place a group of five rats weighing about 45 to 50 gm. on McCollum's diet 3143 for 14 days, at the end of which period the material to be assayed was fed separately to each rat. Two methods were employed in this individual feeding. In the earlier work, the material to be tested was first mixed with a small quantity of the rachitic diet and was then fed to the rats in separate cages and each animal was left in the cage until it had completely eaten its ration. The cod liver oil and earlier stocks of special Acterol were assayed by this feeding method. In the later work, a known concentration of the material in

peanut oil was fed to the animals by means of a stomach tube. This latter procedure seemed to us to be a more accurate measure of the amount actually utilized by the animals. The rats were fed for 10 days on this experimental diet and the bones examined according to the usual line test technic ('22). A therapeutic dose was considered to produce a ++ heal in 10 days.

All the feeding tests involving assay were carried out in a darkened room from which all possible sources of ultraviolet had been eliminated. The animals, when not eating their special diet, were kept in large cages equipped with false bottoms. All cages were thoroughly washed with boiling water and then sterilized in an autoclave once a week. All feed and drinking cups were well washed with hot water daily.

2. Blood calcium and phosphorus methods. Blood calcium and phosphorus were determined on two rats of each group on the fourteenth day of the experiment and again just before the rats were killed on completion of the assay. The blood was drawn by heart puncture, which yielded from 1½ to 2 cc. of blood without seriously affecting the rats. Because of the small volume of blood available, it was necessary to modify the regular methods and to use whole blood instead of serum. For inorganic phosphorus, the Fisk-Subbarow method ('25) was modified so that the blood and reagents were used in one-fifth quantities. For calcium, a modification of the Roe-Kahn procedure ('26) for calcium was devised so that the equivalent of 0.5 cc. of whole blood was used for duplicate estimations. In both determinations, a microcolorimeter was used.

B. Routine procedures in the high dosage experiments

1. The animals were fed either the regular stock diet or the rachitic diet plus a definite quantity of Superacterol per rat per day. This extra ration was fed in three different ways. In the earlier work, it was mixed with the regular stock diet, while in the later work, where it was desirable to use higher

doses, we first used a carefully calibrated medicine dropper which was eventually replaced by a graduated stomach tube.

2. All animals were weighed weekly and the growth curves plotted.

3. The behavior of the animals was observed and a careful record of any irregularities kept.

4. Records were kept of matings and of the weight, size and condition of all litters.

5. Animals sacrificed or found dead were carefully autopsied. The organs of the chest and abdomen were carefully examined grossly. The urinary tract was usually dissected out intact and examined under the dissecting microscope for possible calculi. The following organs were carefully weighed: thymus, heart, lungs, liver, spleen, kidneys, and, in the males, the testes.

6. For microscopic study sections were prepared from the thyroid, thymus, aorta, heart, lungs, liver, stomach, duodenum, spleen, kidneys and gonads.

EXPERIMENTS AND RESULTS

A. Standardization of cod liver oil and Acterol

Our assays showed that 0.3 cc. per rat per day of a solution composed of one part of cod liver oil and twenty-nine parts of peanut oil gave a good (++) cure in 10 days. Control experiments showed that the peanut oil used had no detectable activity. On the basis of the statement that the Acterol used was one hundred times as potent as the cod liver oil, we fed a solution of Acterol in which the Acterol was one one-hundredth the concentration of the cod liver oil fed above. This gave us a cure which was slightly better than ++, but which we believed to be within the limits of biological assay. In another assay on a still more potent solution, we used the stomach tube method and found that the strength of the solution was exactly what it was calculated to be. This latter material was a solution of Mead-Johnson's Viosterol which had a potency of 10,000 D $\pm$ 17 per cent. The studies on the cal-

cium and phosphorus showed that there is a more or less distinct relation between the blood and bone pictures. In severe rickets, on the fourteenth day of the rachitic diet, the calcium varied from 6.5 to 7.5 mg. per 100 cc. of whole blood, while the inorganic phosphorus values ran from 1.5 to 3.0 mg. per 100 cc. After being on a curative ration for 10 days following the 14 days on a rachitic diet, the values were found to range from 8.0 to 9.5 mg. per 100 cc. for calcium, and from 4.0 to 6.0 mg. per 100 cc. for phosphorus. When the test ration was not potent enough to give any cure, the values for calcium and phosphorus became even lower than they were on the fourteenth day of the rachitic diet.

B. High dosage experiments

Series 1. Feeding experiments with high dosages of irradiated ergosterol given to albino rats on a standard normal diet. (a) Eighteen normal albino rats, 5 weeks old, placed on the normal stock diet, were divided into four groups containing about the same number of males and females. Each one of these groups was fed, in addition to its normal diet, either a special dose of Acterol (1:100) or an equivalent amount of non-irradiated ergosterol in peanut oil. The Acterol or ergosterol was fed to each animal individually once each day. These additional diets were made up in the following way:

Group I received a diet calculated to contain one hundred times the therapeutic rat dose of Acterol. In all our calculations we adopted the therapeutic dosage as suggested by Mead Johnson & Co. According to their standards, about 1/80 gm. of cod liver oil per rat per day gives a good ++ line test in 5 days, while Acterol is one hundred times as potent. On this basis the diet of group I was made up by taking 100 cc. of Acterol and diluting it with 91 cc. of peanut oil. We estimated that one standardized drop of this mixture would be equivalent to one hundred times the therapeutic rat dose of Acterol.

Group III received an extra ration of four drops of undiluted Acterol (1:100) per rat per day, which is equivalent to approximately eight hundred times the therapeutic rat dose of Acterol.

Groups II and IV received diets containing unirradiated ergosterol in amounts equivalent to the irradiated ergosterol present in the Acterol of the diets of groups I and III, respectively. Based upon the statement of Mead Johnson & Co., that Acterol contains 50 mg. of ergosterol in 140 cc. of oil, the diet of group IV was 4 drops per day of a solution of 50 mg. unirradiated ergosterol completely dissolved in 140 cc. of peanut oil. Group II received as an extra ration 1 drop of a mixture of 100 cc. of the above solution of unirradiated ergosterol and 91 cc. of peanut oil.

This experiment was continued over a period of 42 weeks (3/4/29 to 1/3/30). The weights of the animals in these four groups showed that growth was fairly normal. No distinct gross pathology was observed. Several of the rats in group I showed pathological changes in the lungs, but this was diagnosed as pneumonia, a very common rat ailment, and could, therefore, not be attributed to the Acterol fed. All the females in group I and one female in group III failed to reproduce which at first led us to believe that a factor of sterility might be involved. This experiment has been repeated many times with animals receiving ten and a hundred times as much of the same lot of Acterol without producing any similar conditions, and we therefore conclude that the failure in reproduction was due to some factor other than Acterol.

(b) The second experiment under this general heading was started with ten 6-month old rats. These animals were fed a normal diet with the exception that the animals in group V received an extra individual ration consisting of ten thousand times the therapeutic rat dose of Acterol, while the four rats of group VI were fed an extra daily ration of unirradiated ergosterol in peanut oil equivalent to the concentration of ergosterol in the diet of the rats in group V. The actual

amount of extra material fed in this case was 7 drops of Superacterol which is six times as potent as Acterol, according to Mead Johnson & Co. assay. This experiment was carried on for a period of 3 years and 15 weeks (4/5/29 to 7/20/32). As the first and second generation animals were obtained they were immediately, on weaning, placed on the same test diet as their parents. In some of the later work the young were fed the test diet directly by means of a medicine dropper beginning at the first or second week after birth.

In summing up the work on these groups, we can say that there was very little if any difference in the growth of the two groups or their offspring. Several of the young in group V died shortly after weaning, but this may be explained by the fact that in these cases the mother had been mated too young and the whole litter was not up to the standard. Furthermore, the later work, when greater care was taken in mating, shows no such picture. Throughout this work no gross or microscopic pathology was observed except that which was due to pneumonia. Even those young which received this large dose of Superacterol when only a week old appeared perfectly normal in all respects upon weaning. Four of these young animals, of the fourth generation, were selected and placed on an extra ration of 15 drops of a new lot of Superacterol which had a potency of 1000 D. These animals received approximately 36,000 times the therapeutic rat dose over a period of 2 years and 21 weeks (2/21/30 to 7/20/32), and seemed to be normal in all respects. The three females all gave birth to normal litters. One of the females was killed during the thirty-first week of the experiment and a careful study of her tissues showed no pathology. During the forty-second week of this experiment the three remaining animals were placed on 20 drops of Superacterol which was equivalent to approximately 50,000 times the therapeutic rat dose. The male in this group was killed in the fiftieth week of the experiment and again the tissues were studied for evidences of pathological lesions with no results. The second female of this series died during the sixty-sixth week of the

experiment and again there were no signs of gross or microscopic pathology. The last animal, female 153, died in the sixty-ninth week of the experiment, while blood was being drawn from the heart by means of heart puncture.

Eighteen of the fifth-generation animals from the litters of the three fourth-generation females were observed over a period of 43 weeks (10/9/30 to 8/25/31), while receiving approximately 36,000 times the therapeutic rat dose and at no time did they exhibit any pathological symptoms. Twelve young animals of the sixth generation were observed under the same conditions over a period of 18 weeks (4/25/31 to 8/20/31) without showing any toxic symptoms. This is the shortest period of observation for any of the animals in this series. The total number of animals in this experiment was 127 and they were divided in the following manner: 10 first-generation adults, 48 second, 35 third, 4 fourth, 18 fifth, and 12 sixth-generation young animals.

(c) The third experiment under this general heading was started with five normal animals 4 weeks of age. These animals were fed a normal stock diet which was made up so that it contained 25 mg. of irradiated ergosterol per 10 gm. of original diet. This ergosterol was irradiated in the dry state by a Cooper-Hewitt mercury vapor lamp; the time of irradiation was 35 minutes at a distance of 40 cm. An accurate record of the food consumption of each rat was kept and it was found that on an average each rat ate 10+ gm. of food per day which meant that each animal was getting 25+ mg. of irradiated ergosterol per day. An assay of this irradiated ergosterol showed that it gave a good ++ cure in 10 days when fed in doses of 0.001 mg. per rat per day. Thus the animals in this experiment were receiving 25,000 times the therapeutic rat dose. These animals were kept under observation for 23 weeks (8/12/29 to 1/24/30), and showed absolutely no evidences of hypervitaminosis. The offspring of these animals were also put on the same diet and observed for 10 weeks (1/13/30 to 3/28/30), and likewise gave no signs of abnormality.

Series 2. Feeding experiments with high doses of irradiated ergosterol given to albino rats on a rachitic diet. (a) The following experiment was designed to study the possible toxic effects of excessive doses of Acterol on animals which were on an otherwise rachitic diet. Sixteen rats, 4 weeks of age, were placed on the McCollum diet 3143 for a period of 14 days. At the end of that time ten of them were fed in addition a quantity of Acterol equivalent to ten thousand times the therapeutic dose. The remaining six rats were fed on a control diet of unirradiated ergosterol in peanut oil. With the exception of female 22 all the animals on Acterol showed a fairly normal growth for the first 5 weeks, although the rate of gain was slightly lower than normal. Female 22, the exception to the above, commenced to lose weight as soon as she was put on the curative diet and finally in the fifth week on curative diet she was killed and an autopsy performed, the report of which is herewith given:

Although this animals had lost 10 gm. in weight (80 to 70 gm.) during the 31 days on the test diet, at autopsy no definite pathology was identified. No calcium deposits were noted in any of the tissues nor were there any proliferative changes or necrosis such as have been described by other workers as preliminary to the pathological calcification which they have reported. No calculi were present in the urinary tract. The great vessels were elastic and were not sclerosed or calcified.

After the fifth week the remaining animals in this group began to show distinct fluctuations in growth. This was true in all cases except that of female 34 which showed a constant though slow gain. Male 28, although he had been gaining weight slowly, suddenly developed a distaste for his food and refused to eat. He next developed a marked diarrhea and an enormous distention of the bladder and finally, having eaten very little for 2 or 3 days, he went into distinct tetanic convulsions in which he died. The autopsy showed these conditions:

This animal had a bladder which measured 20 x 20 x 25 mm. Both ureters were distended and the kidneys were enlarged.

No calculi were found. The kidneys weighed together 2.33 gm., about double the normal weight for this size animal. The spleen seemed abnormally small. The microscopic picture was that of a hydronephrosis. No line of calcification at the junction of cortex and medulla was seen as reported by Dixon and Hoyle ('28). All other tissues were normal.

In order to make a comparative pathological study, female 132 of this group was sacrificed as was also a control female of the same age. Studies by various different staining technics for showing calcium, which will be described in a subsequent paper on the microscopic findings, showed no differences between the two animals which could be classified as pathological.

Male 23 showed a distinctly poor growth curve although his appetite was good. His total gain in weight at the peak of his growth curve in the twenty-third week, was only 49 gm. Besides this he showed a distinct prolapse of the penis. The penis, hanging out from the body, had the appearance of a loose bluish-colored appendage of the skin. The other male in this group, 35, though underweight, seemed to be normal in all respects.

The three animals in this control group on unirradiated ergosterol showed no great differences from the above group. Female 36 which showed an irregular growth curve died during the twenty-second week and the following gross changes were observed:

There was some thinning of the hair especially over the back, moderate emaciation and considerable paleness of the tissues except the lung, which was bright red. The liver showed the lobular markings quite prominently and an area at one margin 5 x 3 x 2 mm. in which the liver tissue appeared pale yellowish and caseous. This was surrounded by a dark hemmorhagic zone about 1 to 2 mm. wide. All other tissues examined appeared normal although no thymus tissue could be identified.

Of the two males in this control group, male 38 exhibited the symptoms listed above of flaccid penis, etc., and male 42

seemed quite normal in all respects except in weight. However, this male showed a better and more consistent gain in weight than any animal in either group.

In reviewing the work on this group which was in progress for 51 weeks (2/27/29 to 2/21/30), we find that all the animals in the control group died except male 42, which seemed to be perfectly normal in all respects. In the group receiving the Acterol all animals except female 34 and male 35 died. These two animals appeared almost normal although they were not quite as sleek looking as our stock animals. Female 34 had one litter of five out of which three died before they were weaned. It is our impression that the irregularities in growth and development in the animals in this group were due to the deficiency of the diet rather than to any effects of the ergosterol feeding.

The most striking observation, however, was the fact that none of the animals, in the control group, which came to autopsy, showed active rickets although they had been on a vitamin D free diet. There are several reasons which might have been the cause of this situation. In the first place, three of the animals in this group died and were eaten by their litter mates before we were aware of their death. In this way the phosphorus and the small amount of residual vitamin D in the tissue of the dead animals was made available to the survivors. Next, those which were autopsied showed a slight loss of weight before death or else had not shown any gain for several weeks. It is known that in order to show a typical rachitic bone picture at autopsy an animal must have shown consistent growth throughout the experimental period. Another factor which may have been of some importance was the fact that after the twenty-third week of the experiment, the animals were not kept in a completely darkened room, although they were never exposed to the direct sunlight. Then, finally, in feeding such a high concentration of ergosterol it might be possible that there was a sufficient contamination by active material to cause the animals to show a normal bone picture.

Series 3. Feeding experiments with extremely high doses of irradiated ergosterol given to albino rats on a normal diet, with and without potassium iodide, and on a rachitic diet without iodide. (a) The negative results which were obtained in the above experiments led us to start an experiment in which extremely large doses were given to the animals in an effort to see whether we could obtain any of the toxic symptoms reported by others. In this series, we used the Mead-Johnson Viosterol which had a potency of 10,000 D. All test materials were fed by stomach tube. We started with fifty-two albino rats which were divided in the following manner:

Group VII. Twenty-seven animals received the normal stock diet plus 93,000 times the dosage of Viosterol.

Group VIII. Twenty-five animals received as an added daily ration 93,000 times the therapeutic rat dose of Viosterol and also various amounts of potassium iodide. The iodide was added in this case because of the claims made by Simonnet and Tanret ('30), who maintained that the addition of large quantities of iodide prevent the calcification due to excessive doses of irradiated ergosterol.

Group IX. Twenty controls, ten of which received a quantity of peanut oil equivalent to the amount received by the experimental animals, and the other ten received an amount of potassium iodide equivalent to the amount fed to the test animals.

This experiment was continued over a period of 37 weeks (11/6/31 to 7/21/32). As far as the gross condition of the rats is concerned, the feeding of the iodide had neither a deleterious nor advantageous effect. The animals seemed normal in all respects and no gross pathology was observed.

It was, however, felt desirable to carry this experiment on a little further, so three of the healthiest males of group VII were taken and fed an amount of Viosterol equivalent to the 465,000 times dosage. Some very striking results were now obtained. Two of these animals died on the fifth day of feeding and the third showed a distinct loss in weight, a

general emaciation, a slight diarrhea and a definite loss of appetite with an associated general apathy. Two of the animals showed a distinctly bloody urine and the last animal to die showed very marked evidences of calcification in the aorta. This striking finding led to a continuation of the experiment and this was done by starting nine young animals on this high dosage. In this case three animals died during the first week, four during the second week, and the rest during the third week.

(b) In order to satisfy ourselves as to the validity of these results we next treated twenty-seven normal adults as follows:

Group X. Thirteen normal females, 9 months old, who had all had one normal litter, were so divided that nine of them received the 465,000 times dosage of Viosterol, while the remaining four received the 93,000 times dosage.

Group XI. Seven normal females, and seven normal males, all 3 months of age, were so divided that five females and six males received the 465,000 times dosage, while the balance of two females and one male received the 93,000 times dosage of Viosterol.

All animals on the 465,000 times dosage died within 5 weeks; 11 females and 2 males in the first, 2 females and 3 males in the second, 1 male in the fourth and 1 female in the fifth week. All developed a slight diarrhea, loss of weight, a flabby emaciated musculature, inactivity and loss of appetite. Most of them showed a bloody nasal discharge which appeared to be associated with a difficulty in breathing. All of the rats on the 93,000 times dosage survived during this period although some of them did not appear to be normal and had lost considerable weight. The blood from several of these animals was analyzed for calcium and inorganic phosphate, and no significant rise was noted, which is in agreement with the report of Jones, Rapoport and Hodes ('30).

(c) A third experiment under this general heading was now started and this involved the use of eighty-four normal animals of approximately 4 weeks of age. These animals were divided into three major groups each of which was again

subdivided into three smaller groups according to the following scheme:

Group XII. Thirty animals were placed on a rachitic diet for a period of 2 weeks, at the end of which time they were kept on the same basic diet and received in addition 46,500, 93,000 and 465,000 times the therapeutic rat dose of Viosterol, respectively.

Group XIII. Twenty-seven rats were placed on a rachitic diet for a period of 14 days at the end of which time they were placed on a normal diet and divided into the three sub-groups listed above.

Group XIV. Twenty-seven animals were placed on a normal stock diet for a period of 2 weeks, at the end of which time they were divided into the three sub-groups referred to above, but continued on the normal basal stock diet.

In this experiment, we wished to determine three things, 1) were the animals more resistant to this toxicity when on a McCollum vitamin D free diet than when on a normal diet; 2) what was their reaction when rachitic and then replaced on a normal diet plus the overdosage of irradiated ergosterol; and, 3) the difference between the various levels fed under the different conditions of the experiment. The results obtained under these conditions were quite clear cut and definite. All of the animals receiving the 465,000 times dosage with McCollum's vitamin D free diet were dead by the sixth day following the first dose of irradiated ergosterol. These animals all showed evidences of emaciation with an extensive loss of weight, all exhibited signs of diarrhea and all had a bloody discharge from the nose. At autopsy no gross pathology of the organs was observed. The animals receiving the 93,000 times dosage did not show the extreme symptoms listed above, but they did show a steady loss of weight and by the end of the third week were so weak that they could hardly move about. All of these animals died or were killed during the fourth week. Some of these animals had a slight diarrhea and all had a more or less bloody nose. The main gross autopsy findings were that in all but two of the animals the

liver and kidneys were a pale grayish brown color speckled with darker brown dots. In female 500 the lung was full of caseous nodules while in male 497 the spleen was very friable. The animals receiving the 46,500 times dosage appeared normal in growth and appearance for the first 2 weeks, and then all but four of them also began to lose weight, although their loss was only gradual. These animals were all killed during the fifth week of the experiment and we were unable to observe any gross pathology other than the loss of weight.

In the group of animals which was transferred from the rachitic to the normal diet at the end of the second week, several characteristic findings were observed. Of the animals receiving the 465,000 times dosage, two died during the first week, seven during the second week and the remaining animal died during the third week of the experiment. Here again the animals had lost much weight and at autopsy we observed that the kidneys were of a very pale grayish brown color, speckled with darker brown dots, and on cutting through the kidneys it was seen that this was the color of the entire cortex. The thymus in all these animals was quite small and the adrenals appeared larger than those of normal animals. The hearts of these animals showed a yellowish mottling against the background of the darker myocardium. The animals which were receiving the 93,000 times dosage appeared quite normal during the first week of the experiment and with the exception of two animals which lost slightly in weight, appeared quite normal during the second week. All of these rats were killed during the third week of the experiment and no gross evidences of pathology were observed. Those animals receiving the 46,000 times dosage appeared perfectly normal and when killed during the third week showed absolutely no symptoms of any gross pathology.

The groups receiving a normal diet from the time of weaning, and which were 2 weeks later placed on the 46,500 and 93,000 times dosage were normal in all respects and at autopsy during the third week gave no evidences of any gross pathol-

ogy. The animals receiving the 465,000 times dosage again showed all the symptoms listed in the previous two groups on this level of Viosterol, namely, pale speckled kidneys, mottled heart, small thymus and large adrenals. All but one of these animals were dead by the end of the second week of the experiment and this animal was killed during the third week. The arch of the aorta of this animal (male 564) showed signs of calcification. In order to assure ourselves that we were observing all the gross pathology, we killed two groups of three animals each, consisting of litter mates on the three levels of irradiated ergosterol fed. The tissues of these animals were then compared grossly with each other and with the tissues of a normal untreated animal of the same age. When this was done it was seen that the thymus of the animals on the 465,000 times dosage was less than one-fourth the weight of the thymus in the other treated animals and in the normal control. In fact, in the subsequent animals brought to autopsy this relationship was in most cases much more pronounced. This comparative study also emphasized the previously noted difference in the size of the adrenals and also definitely brought out the fact that the myocardium of the highest dosage animals was affected in some manner by the production of slightly elevated areas of a yellowish mottling which did not appear to correspond to the major vessels of the myocardium. Furthermore, this study substantiated our belief that, at least from a consideration of gross pathology, the animals receiving the 46,500 and the 93,000 times dosage were in all respects normal. Finally, in order to determine whether the change in the thymus was an atrophy or merely a cessation of growth, several normal animals, of the age of the experimental ones at the time when they were first fed ergosterol, were killed and it was observed that in all these cases the thymus of the untreated animals was two to three times larger than the thymus of the highest dosage animals and that in all instances the thymus was smaller than the thymus of the animals receiving the 46,500 and 93,000 times dosage, respectively, or than the thymus of the older normal controls.

Series 4. Experiments on the protection of the young against rickets. With so many young animals available from the other experiments in progress, we thought it would be interesting to check the findings of Bills and Wirick ('30) as to the influence of activated ergosterol in the mother's diet on the resistance of the young to rickets. Seventy-six animals were used in this work. These animals were from mothers receiving 10,000 times the therapeutic rat dose of irradiated ergosterol and were placed on McCollum's rachitic diet 3143 at weaning. At the end of a 3-week period the animals were killed and studied by the line test and blood analyses for rickets. In all cases, there was only a slight degree of rickets.

Another experiment involving thirty-seven rats was run. In this experiment the mothers were receiving an extra ration of 10,000 times the therapeutic rat dose of irradiated ergosterol and immediately after birth the young were given to a foster mother on normal diet. All of these animals showed the characteristic rachitic blood analyses and line test after having been on the rachitic diet for 3 weeks. These results definitely confirm the findings of Bills and his co-workers ('30).

DISCUSSION

From the data presented it seems clear that rats when fed a normal bread-milk diet are not affected by irradiated ergosterol if given 46,500 times the therapeutic rat dose. When similar animals are given 93,000 times the therapeutic rat dose of irradiated ergosterol some toxic symptoms are observed. The effect of this dosage is, however, quite different on young and adult animals. When young animals are given this amount of irradiated ergosterol, they show no immediate symptoms; in fact, they develop quite normally and it is only as they approach the age of 9 to 12 months that they begin to manifest any symptoms of toxicity and these are not extremely serious, having in no case in our series led to the death of the animal. When 93,000 times the therapeutic rat dose of irradiated ergosterol is fed to animals 3 and 9 months old, respectively, there is an immediate loss

in weight which is much more pronounced in the older animals. In some cases this dosage led to death of the animals with the same gross pathological findings as are observed in animals on a higher dosage. It is quite significant to note that there is a definite difference in the reaction of different adult animals when on this level of irradiated ergosterol. This is very strikingly brought out in the seven rats on this dosage in groups X and XI; of these, two rats, 675 and 619, died while the remaining animals appeared quite normal except for slight loss in weight. This experiment also shows the difference between the reaction due to age, for the animals which were 3 months of age lost very little weight and none of them died while the 9-month-old animals lost weight steadily and two of them died.

When 465,000 times the therapeutic rat dose of irradiated ergosterol was fed to either young or adult animals there was an almost immediate toxic response. This reaction was characterized by the following symptoms: immediate loss of weight, the appearance of diarrhea and of a bloody discharge from the nose, the development of a muscular flabbiness, a definite loss in appetite and a very marked emaciation. When brought to autopsy these animals showed definite gross pathology. There were varying degrees of calcification of the aorta, the kidneys were a very pale grayish color and speckled with darker brown dots, the thymus was atrophied, the adrenals were larger than in the normal, and the myocardium showed a grayish yellow mottling. Here again we can see the greater susceptibility of the adult animals to these high dosages. Seventeen of the adult animals fed on 465,000 times the therapeutic rat dose of irradiated ergosterol and a normal diet were dead by the end of the fifth day, one lived for 2 weeks, while the last two rats lived for 3 weeks. Of the nineteen young animals on this level of irradiated ergosterol and a normal diet only three were dead at the end of the first week while the other sixteen died or were killed between the thirteenth and seventeenth days of the experiment.

Certain workers (Kreitmair and Hintzelmann, '28; Krietmair and Mell, '28, and Pfannenstiel, '27, '28) have also reported the atrophy of the spleen to be one of the results of excessive doses of irradiated ergosterol, but we could not confirm this finding. The weights of the spleens in our animals varied considerably and at times we thought we were confirming this finding. However, when we selected the records of all the animals receiving irradiated ergosterol for which there was a control rat of approximately the same weight, we found that the total weight of the spleens of the animals receiving irradiated ergosterol was 0.37 per cent of the total gross weight, while the total weight of the spleens of the control animals was 0.38 per cent of the totol gross weight. From these facts we feel justified in saying that we have not the slightest evidence of any splenic atrophy in our rats. Perhaps the most consistent and outstanding pathology we observed was in the thymus. In the eighty-four animals in groups XII, XIII and XIV, the sixty-five animals on the 46,500 and 93,000 times dosages of irradiated ergosterol as well as four normal controls, had a thymus anywhere from three to four or more times as heavy as any of the animals on the 465,000 times dosage. In fact, in many cases the thymus of the highest dosage animals was so small as to be almost unrecognizable and unweighable.

Many investigators have reported the finding of calculi in the urinary tract which have given rise to hydronephrosis. In our series, we had one rat receiving 10,000 times the therapeutic dose of irradiated ergosterol and McCollum's rachitic diet 3143 which developed hydronephrosis due to an obstruction in the bladder or lower down. We were, however, unable to locate a calculus although it may have been present. Another rat had a mild distention of the bladder and a small calculus 1 mm. in diameter was found at the urinary trigone. However, the largest calculi we found were in a control animal on a rachitic diet. There were two of them, each of which measured 3 x 3 x 6 mm. They were crystalline in structure and gave a strong test for calcium, but only a very faint phosphate test.

Brown and Shohl ('30) claim that animals on a rachitic diet and receiving large overdoses of irradiated ergosterol (Vigantol) are more resistant to the toxic effects than animals on a normal diet. We cannot subscribe to this claim, for in our work we found that the animals on a rachitic diet showed the toxic effects much more rapidly and at a lower level than those on a normal diet. Of the animals in group XII, the ten animals receiving McCollum's rachitic diet 3143 and 465,000 times the therapeutic rat dose of irradiated ergosterol were all dead by the end of the first week, and the ten animals receiving the same diet and 93,000 times the therapeutic rat dose of irradiated ergosterol either had died or were killed by the end of the fourth week because of their extremely weak condition. In contrast to this only three of the animals in groups XIII and XIV receiving the 465,000 times dosage died during the first week, while all but two of the remainder died or were killed during the second week and the last two during the third week. Furthermore, of the eighteen animals in these groups receiving the 93,000 times dosage, only two animals showed a very slight loss of weight at the end of the second week and these two animals were from group XIII and had been on a rachitic diet during the first 2 weeks of the experiment. The other animals were normal in all respects. The difference in these findings may be due to the fact that the rachitic diet used in our work was a much more severe diet than that used by Brown and Shohl. However, in their first paper ('30) they compare animals which had been on a rachitic diet plus irradiated ergosterol for 6 days with animals which had been on a normal diet plus the same amount of irradiated ergosterol for 11 days and we feel that such a comparison is not warranted. In their second paper ('30) these workers do not present enough clear cut experimental evidence to uphold their contention that on a rachitic diet the toxic effects are not as pronounced as on a normal diet.

A detailed discussion of the gross and histological specimens of all the experimental animals will appear in a later publication.

SUMMARY AND CONCLUSIONS

1. Commercial preparations of irradiated ergosterol of exceptional potency were standardized by the McCollum line test and found to possess the claimed activity.

2. Ten albino rats were placed on a stock diet plus 100 to 800 times the therapeutic rat dose of this preparation for 36 weeks without observing harmful effects.

3. The progeny of 48 second, 35 third, 4 fourth, 18 fifth and 12 sixth generation rats were placed on the same stock diet plus doses up to 50,000 times the curative dose without observing harmful effects. The sixth generation animals were treated for 18 weeks, all others were treated for longer periods.

4. Pure ergosterol was irradiated in the solid form by a Cooper-Hewitt mercury arc lamp and fed to albino rats and their offspring in amounts up to 25,000 times the curative dose for 10 to 23 weeks without harmful effects.

5. Albino rats were placed on the McCollum 3143 diet with and without the commercial preparation in amounts up to 10,000 times the therapeutic rat dose. Although most of the animals died in less than 30 weeks, no true hypervitaminosis could be detected.

6. Young and adult albino rats were kept on a stock diet including bread and milk, plus 46,500 times the therapeutic rat dose for 3 weeks without harmful effects.

7. Young albino rats were placed on a stock diet including bread and milk plus 93,000 times the curative dose for 12 months when some of them showed loss of weight. Adult animals under the same conditions showed immediate loss of weight and in some cases died with pathological findings similar to those indicated below.

8. Young and adult albino rats were placed on a stock diet including bread and milk plus 465,000 times the therapeutic rat dose with the appearance of immediate toxic effects. There were immediate loss of appetite and weight, bloody discharge from the nose, diarrhea, marked muscular flabbiness, and gross pathological changes involving enlarged supra-

renals, atrophied thymus and abnormal appearance of the kidney and heart. The adult animals were again more susceptible than the young ones.

9. Rachitic diet was substituted for the stock diet on the 46,500, 93,000 and 465,000 dosage experiments on young animals. This change made the animals more susceptible.

10. Potassium iodide was added on the dosages of 93,000 times the curative amount without changing the picture.

11. Young from mothers on 10,000 times the therapeutic rat dose were weaned and placed on the rachitic diet. Only very slight rickets developed. Similar young, but nursed by stock diet foster mothers, developed typical rickets.

12. These results indicate that the toxic effects observed by others on relatively low dosages of irradiated ergosterol are due to the presence of a toxic substance other than the true antirachitic agent in appreciable amounts in the preparations tested. Whether the toxic effects observed by us on our commercial preparation when given in the very high doses are due to a trace of this hypothetical by-product or to the vitamin D substitute remains to be determined.

13. Our results again indicate that the toxicity of a given irradiated ergosterol preparation is also determined in part by the character of the diet. In general, the more complete and better balanced diets act in a more protective manner.

ACKNOWLEDGMENTS

We wish to express our sincere thanks and appreciation to Dr. F. C. Koch and Dr. R. G. Gustavson for their kind help and advice during this investigation.

Thanks are also due Dr. George F. Harsh and Dr. H. C. Huston for their help in the preparation and diagnosis of the pathological material.

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FACTORS WHICH DETERMINE RENAL WEIGHT

XVI. THE NATURE OF THE PROTEIN INTAKE

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ONE FIGURE

(Received for publication December 13, 1933)

The protein intake has been found to be (Mac Kay, Mac Kay and Addis, '28 a, '28 b; Mac Kay and Mac Kay, '31 a, '31 b) one of the most important of the factors which determine the weight of the kidneys in the albino rat. The relationship between the protein intake and renal weight when casein is the protein, may be expressed for male rats of all ages by the empirical formula,

$$\text{renal weight} = (\text{protein intake} + 2.75) \div 0.0183$$

In the study reported here we were interested in whether or not this relationship was limited to casein or held generally for natural protein products.

Four sources of protein, namely, crude commercial casein, commercial 'egg albumin,' commercial 'blood albumin' and gelatin were used. Except for gelatin these were fed separately. For reasons detailed later gelatin was fed in combination with casein. The method of experiment was identical with that used before (Mac Kay and Mac Kay, '31 a). A group of about twenty male albino rats were fed upon each diet from 26 to 70 days of age. The diets which were used are described in table 1.

Growth on the various diets was interesting. In table 2 are depicted the percentage changes in body weight for each

TABLE 1

INGREDIENT	DIET											
	1A	1B	1C	2A	2B	2C	3A	3B	3C	4A	4B	4C
Casein	16	46	75							16	16	16
Egg albumin				16	46	74						
Blood albumin							16	46	74			
Gelatin										8	30	50
Cornstarch	45	20	1	45	20	2	45	20	2	37	20	5
Lard	15	10	0	15	10	0	15	10	0	15	10	5
Cod liver oil	9	9	9	9	9	9	9	9	9	9	9	9
Salt mixture	4	4	4	4	4	4	4	4	4	4	4	4
Dried yeast	9	9	9	9	9	9	9	9	9	9	9	9
Agar-agar	2	2	2	2	2	2	2	2	2	2	2	2
Per cent of diet												
Protein	18	43	67	17	41	64	17	42	66	23	44	62
Fat	24	20	11	24	20	11	25	20	11	24	20	16
Carbohydrate	42	20	3	43	22	5	42	21	4	37	20	6
Water	6	6	7	6	6	8	6	6	7	6	6	6
Roughage	5	5	5	5	5	5	5	5	5	5	5	5
Salts	5	6	7	5	6	7	5	6	7	5	5	5
Per cent of calories												
Protein	16	40	71	15	38	68	15	39	70	20	40	60
Fat	48	41	26	48	42	26	49	42	26	47	41	34
Carbohydrate	36	19	3	37	20	6	36	19	4	33	19	6

TABLE 2

GROUP	WEIGHT 26 DAYS OLD	40 DAYS OLD	50 DAYS OLD	60 DAYS OLD	70 DAYS OLD
	gm.	*Percentage of weight at 26 days*			
1 A	42	193	262	348	426
1 B	42	207	305	393	469
1 C	39	195	277	354	423
2 A	42	195	279	369	444
2 B	44	177	211	245	259
2 C	45	175	187	195	200
3 A	45	191	255	318	317
3 B	48	206	299	393	398
3 C	45	155	213	271	284
4 A	42	190	211	314	328
4 B	52	175	225	295	321
4 C	44	157	169	181	193

group. The increase in body weight was greatest on the casein diets. The 'blood albumin' diets also gave good growth. In both cases the 46 per cent protein diets were optimal. Both the egg albumin (with the exception of the diet with the lowest concentration) and the gelatin diets gave poor growth and the higher the concentration of these proteins the worse the increase in body weight. The significance of the weight changes is borne out by a comparison of the average body lengths (table 3) at death.

Numerous rats receiving the egg white diets developed the 'pink disease' described by Boas ('27) and Findlay and Stern ('29) as occurring when raw egg white (as our product was) is fed to rats. Except for the loss of hair and the thickening and redness of the skin there were no other symptoms and the animals appeared to be in fairly good condition. The more serious symptoms noted by Parsons ('31) were not observed. The affected rats were distributed throughout all three diet groups so that the occurrence of this 'egg white disease' in its mild form was probably without influence upon the results.

We have noted the incorporation of casein in the gelatin diets. Some years ago an attempt was made to feed rats on diets containing gelatin as the sole source of protein. A commercial[1] powdered gelatin was used. Growth was poor but the animals were really in very good shape until they suddenly died in uremia. Further examination disclosed a marked proteinuria before this time and at autopsy the kidneys were twice their normal size and weight. They were firm and gray in color. Histological examination disclosed extreme epithelial degeneration. Later on these findings followed the feeding of a purer gelatin.[2] These brittle sheets were difficult to pulverize and casein was incorporated in one diet to overcome this mechanical difficulty. This diet failed apparently to damage the kidneys. The question of the protection against renal damage from ingested gelatin by the

[1] 'Delft' brand.
[2] 'Gold Label' brand.

TABLE 3

DIET NO.	NUMBER OF RATS	BODY WEIGHT	BODY LENGTH	BODY SURFACE	HEART WEIGHT		LIVER WEIGHT		KIDNEY WEIGHT				RATIO: BODY LENGTH / BODY WEIGHT
					Actual	Per 100 sq. cm. body surface[1]	Actual	Per 100 sq. cm. body surface[1]	Actual	Per 100 sq.cm. body surface[1]			
										Mean	P.E.	Coef. Var.	
		gm.	*mm.*	*sq.cm.*	*mg.*	*mg.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*per cent*	
1 A	25	170	192	348	640	184	8.42	2.42	644	185	1.4	6.0	0.89
1 B	21	195	198	381	730	191	8.69	2.28	832	218	2.0	6.9	0.99
1 C	29	159	189	332	644	194	9.07	2.73	964	289	1.8	5.0	0.84
2 A	22	180	194	361	656	183	7.54	2.24	643	178	1.6	6.1	0.93
2 B	24	110	172	259	434	168	5.12	1.98	607	235	2.5	7.6	0.64
2 C	20	86	162	219	347	158	3.95	1.80	585	268	3.0	7.4	0.53
3 A	20	138	186	301	516	171	7.78	2.58	607	202	2.5	7.6	0.74
3 B	22	197	200	384	676	176	9.92	2.58	1054	262	2.5	6.6	0.98
3 C	20	133	181	295	533	181	8.52	2.89	905	307	2.3	4.9	0.74
4 A	20	129	182	289	503	174	5.91	2.04	584	201	3.4	11.9	0.71
4 B	22	173	193	351	648	184	9.16	2.61	1137	322	4.8	10.4	0.90
4 C	21	109	170	258	433	168	6.58	2.55	1148	433	5.3	8.2	0.64

[1] Body surface calculated by the formula of Carman and Mitchell ('26)—S = 11.36 W 2/3.

addition of casein to the diet is now the subject of another investigation. In the present experiments the kidneys appeared normal when the rats were killed. Likewise there was no evidence of any renal pathology upon any of the other diets.

The data for the twelve experiments have been summarized in table 3. Among the incidental measurements the small livers in the egg albumin fed rats, particularly on the higher intakes, are noteworthy. On every diet the kidneys became larger when the amount of protein was increased. The kidney weights produced by the gelatin diets are far beyond any of the others and although this may be a specific physiological effect of the gelatin we are prone to believe that it is an early stage in the renal damage from gelatin that has been mentioned. True, the blood urea concentrations of these gelatin fed rats were not abnormal and by routine histological methods no changes were noted in their kidneys. However, the swelling of the epithelial cells which eventually goes on to necrosis is very difficult to discern in the early stages.

In table 4 under the 'A' columns the kidney weights as observed in relation to the body surface calculated by the formula of Carman and Mitchell ('26) have been compared with those calculated from the average protein intake (in relation to the same body surface figure) during the last 10 days of each experiment by means of the casein formula:

$$\text{renal weight} = (\text{protein intake} + 2.75) \div 0.0183$$

Both the egg albumin and the blood albumin diets apparently produced kidneys significantly larger than would be expected from the protein intakes. This extra enlargement may be a pseudo-enlargement due to our method of calculating body surface. Carman and Mitchell's formula was perfectly satisfactory for rats in good condition or where the body surface simply served as a reference standard for the kidney weight and food ingredients in a given group of rats as in earlier studies in this series. When it is desired to compare the relationship with the standard in diverse groups as in the present study, it may not be satisfactory. In our calculations

TABLE 4

GROUP NO.	PROTEIN	INTAKE PER 100 SQ.CM. BODY SURFACE PER DAY				MILLIGRAMS KIDNEY PER 100 SQ.CM. BODY SURFACE				DEVIATION OF OBSERVED FROM CALCULATED				RATIO: KIDNEY WEIGHT / HEART WEIGHT
		Food		Protein		Observed		Calculated		A	B	B^1	B^2	
		A	B	A	B	A	B	A	B					
		gm.	gm.	gm.	gm.					per cent	per cent	per cent	per cent	
1 A	Casein	3.39	4.40	0.61	0.79	185	240	183	193	1.8	24.3			1.01
1 B	Casein	3.02	4.06	1.30	1.74	218	293	221	245	—1.4	16.4	17.6	0.0	1.14
1 C	Casein	3.71	4.76	2.49	3.19	289	370	286	325	1.5	12.2			1.50
2 A	Egg albumin	2.59	3.35	0.44	0.57	178	232	174	181	2.2	22.0			0.98
2 B	Egg albumin	2.32	2.83	0.95	1.15	235	284	202	213	14.0	25.0	19.9	2.3	1.40
2 C	Egg albumin	2.93	3.44	1.89	2.22	268	311	254	271	5.2	12.8			1.68
3 A	Blood albumin	2.14	2.62	0.36	0.44	202	247	175	174	7.8	42.0			1.18
3 B	Blood albumin	3.09	4.04	1.30	1.70	262	360	222	243	15.3	48.2	40.9	23.3	1.56
3 C	Blood albumin	3.68	4.54	2.44	3.04	307	420	284	317	10.8	32.5			1.70
4 A	Gelatin	2.53	3.07	0.60	0.73	201	246	183	190	9.0	29.5			1.16
4 B	Gelatin	3.29	4.26	1.43	1.85	322	418	229	251	28.8	66.6	56.0	38.4	1.76
4 C	Gelatin	3.42	3.67	2.19	2.35	443	478	270	278	39.2	72.0			2.65

REMARKS: A = Body surface calculated by the formula of Carman and Mitchell ('26). B = Body surface calculated by the formula of Lee and Clark ('29). B^1 = Averages for the three groups on each type of protein. B^2 = Averages for the three groups on each type of protein adjusted to the basis of zero deviation of observed from calculated.

body surface depends solely on body weight and the body surface: body weight relationship may be changed by variations in the amount of body fat which seemed to exist or severe stunting which is evidenced by the depressed body length—

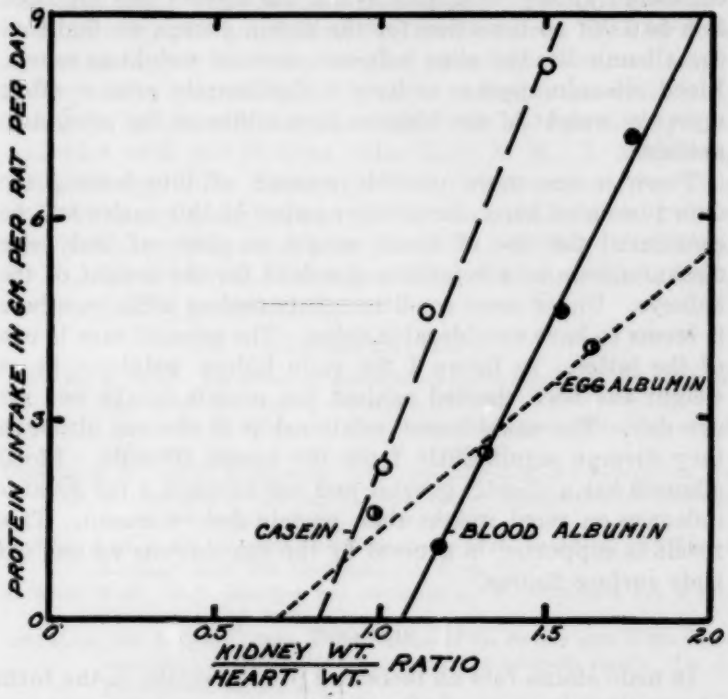

Figure 1

body weight ratios (Jackson, '24) in table 3. Lee and Clark ('29), in their rat body surface formula no. 4, have incorporated the nutritional correction factor used by Cowgill and Drabkin ('27) in their body surface formula for the dog. This formula makes a good correction for variations in body fat and the effects of severe stunting. It is:

$$S = 10.76\ W^{2/3}\ (0.310 + \frac{W^{1/3}}{L})$$

We have recalculated our data on this basis in table 4 under the 'B' columns. Since our protein-renal weight formula is based upon a different body surface even the casein diet groups show a definite deviation of the observed from the calculated kidney weights. When the figures are adjusted to a basis of no deviation for the casein groups we find that egg albumin has the same influence on renal weight as casein. Blood albumin appears to have a significantly greater effect upon the weight of the kidneys than either of the other two proteins.

There is one more possible manner of interpreting the data presented here. In another paper of this series will be considered the use of heart weight in place of body size measurements as a reference standard for the weight of the kidneys. Under some conditions it is useless while in others it seems to have considerable value. The present case is one of the latter. In figure 1 the ratio kidney weight ÷ heart weight has been charted against the protein intake per rat per day. The usual linear relationship is obvious although they diverge significantly from the casein formula. Blood albumin has a slightly greater and egg albumin a far greater influence on renal weight than protein fed as casein. This result is supported in general by the calculations on our old body surface figures.

SUMMARY

In male albino rats an increased protein intake in the form of egg albumin or blood albumin has a greater influence upon renal weight than protein in the form of casein. Gelatin produces a much larger increase in renal weight than any other protein source which has been fed. There is evidence that this may be of a pathological rather than a physiological nature.

ADDENDUM

Shortly after this manuscript was submitted for publication a paper by Wilson (Wilson, H. E. C., Biochem. J., 1933, vol. 27, p. 1348) on the same subject appeared. He reports that gelatin produces a much greater increase in renal weight than other proteins, a finding which we confirm. Wilson does not suggest that this may be of a pathological nature as we believe. His conclusion that glycine and glutamic acid nitrogen have practically the same influence as casein nitrogen coincides with our findings (Mac Kay, E. M., J. Nutrition, 1933, vol. 6, p. 157). This author's findings in connection with the addition of phosphate to the diet must be modified by the fact that phosphate renal enlargement is definitely (Mac Kay, E. M., and Oliver, J., Proc. Soc. Exp. Biol. and Med., 1930, vol. 28, p. 324) pathological.

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THE UTILIZATION OF ENERGY PRODUCING NUTRIMENT AND PROTEIN AS AFFECTED BY INDIVIDUAL NUTRIENT DEFICIENCIES [1]

I. THE EFFECTS OF CYSTINE DEFICIENCY

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TWO FIGURES

(Received for publication January 17, 1934)

INTRODUCTION

The study to be discussed is the first unit in a program of investigations of the methods by which individual nutrients contribute to the specific effects of foods.

The program as a whole and the point of view are direct consequences of the studies on energy metabolism initiated by Armsby in 1902, carried on by him until his death in 1921, and continued since 1922, with many changes of understanding and methods, under the direction of E. B. Forbes.

The fundamental principle upon which this program of studies is based is the net energy conception, in the light of which the net energy useful in nutrition is the gross energy of the food minus the several energy losses and expenses of food utilization.

The principal losses are the energy of the feces and urine, and, in the case of ruminants, the energy of the methane produced by fermentation of carbohydrates; and the energy expenses of food utilization are the work of prehension, mastication, deglutition, fermentation, digestion, peristalsis, ab-

[1] Publication authorized by the Director of the Pennsylvania Agricultural Experiment Station, January 8, 1934, as Technical Paper no. 627.

sorption, transportation, stimulation, synthesis, secretion and excretion.

This simple conception, that gross energy minus expense and loss of utilization equals net energy, has been most attractive, since it has seemed to offer the promise of a fundamentally valid system of measurements of energy values of foods, which would be serviceable as the basis of practical standards of nutrition; and the tables of starch equivalents computed by Kellner ('05), and of net energy values, either experimentally determined or computed by Armsby ('17), were founded upon this idea.

Later developments within the field of nutrition, however, have raised questions as to the practicability of net energy values of individual foods; have revealed them as remainders —varying in response to many influences—rather than as directly determinable constants; and have led this institute to undertake the analysis, which has been in progress for several years, of the whole subject of conditions affecting the utilization of food energy.

In this relation Forbes ('33) recently formulated the law of maximum normal nutritive value, a principle which was by no means new, but which had not been as clearly appreciated nor as generally recognized as it should be, in research in nutrition. This principle signifies, in most relations, that individual foods express their full nutritive value only as they are presented in nutritively complete and perfect, and quantitatively sufficient diets.

In this same light Forbes observed, several years ago ('29), that any deficiency of any essential nutrient must eventually affect the energy value of a ration.

Such sweeping postulates, however, require a deal of proving; hence the undertaking of the series of studies of which the present is the first to reach publication, the object of which is to determine the effects of deficiencies in the supply of single essential nutrients, other than as used for energy production, on the energy value of diets.

The immediate objective of the present study was to compare two diets of the same gross energy value, and of the

same composition, except for a difference in the quantity present of the amino acid cystine.

The criteria on which conclusions depend are the results of digestion, metabolism, and growth experiments—involving the determination of the nitrogen and the gross energy of the food, the feces, the urine and the body increase.

The first important work on the biological significance of the amino acid cystine was that of Osborne and Mendel ('15), who demonstrated that the addition of cystine to casein notably lowered the casein requirement for normal growth of rats.

Lewis ('17) found that a small quantity of cystine added to a diet which was poor in protein (supplied as beef heart) favorably influenced nitrogen retention by dogs.

Sherman and Merrill ('25) reported that cystine is also the first limiting amino acid as affecting the utilization of whole milk protein.

Mitchell and Beadles ('30) demonstrated that a diet containing 8 per cent protein (from skim milk powder) is definitely improved by the addition of 0.24 per cent of cystine, as determined by increase in live weight and in nitrogen retention. They did not determine the gross energy of the rat bodies, but the weight of the ether extract of the bodies indicated that in the case of at least one pair of rats among nine the energy stored by the rat which received the cystine-supplemented diet was less than that stored by its pair mate.

EXPERIMENTAL SUBJECTS

The subjects of the experiments were albino rats, approximately 28 days of age, and selected to weigh as nearly as possible 40 gm. each.

Ten such rats were killed and analyzed, at the beginning of the experiments, to serve as controls, by representing the initial status, as to nitrogen and energy content, of the rats which were fed.

The nitrogen content of the control rats averaged 25.2 mg. per gram of live weight (coefficient of variation = 3.4 per cent); and the energy content per gram of live weight averaged 1.50 Cal. (coefficient of variation = 8.4 per cent).

Expressed on the basis of empty weight (contents of alimentary tract removed), the values of nitrogen and energy per gram were 27.7 mg. and 1.65 Cal., respectively.

The 'fill' (alimentary content) of the control rats averaged 3.66 gm. per rat, the empty weight being 90.85 per cent of the live weight. The computation of the initial nitrogen and energy content of the experimental rats may obviously be made from either the live weights or the computed empty weights with identical results.

A PRELIMINARY EXPERIMENT

In a preliminary experiment in which two groups, of five rats each, were individually fed equal quantities of two rations, one cystine-deficient and the other cystine-supplemented, during a period of 10 weeks, the energy gains by the rats which received the cystine-supplemented diet averaged 14.1 per cent of the gross intake, while the energy gains of the other group averaged 10.6 per cent of the food energy. No difference in the digestibility of the dry matter of the rations of the two groups of rats was found. Since the constituents of the urine were not determined, no values for metabolizable energy and heat production were obtained. The average gain in live weight of the rats which received the cystine-supplemented diet was 71 gm., while that of their pair mates, which received a low-cystine diet, was 50 gm.

A SECOND EXPERIMENT

Following this preliminary test it was determined to conduct a second experiment with the same rations, with a larger number of rats, and to measure also the energy in the feces and urine, thus to learn, more in detail, the differences which characterize the energy utilization of the amino-acid deficient diet.

Ten pairs of weanling rats were selected for this experiment, and were fed for 14 weeks by the paired-feeding method, which was originally proposed by Armsby, but has been advocated of late especially by Micthell (Mitchell and Beadles,

'30). All pair mates were of the same sex, of the same litter, and of approximately the same live weight.

METHOD OF FOOD ASSIGNMENT

Throughout the experiment the quantity of food given to each pair was determined as the quantity consumed, ad libitum, by the rat which ate the less; and the intake of the rats of each pair was exactly equalized at the end of each week.

This procedure has the effect to place the animals which receive the more efficient ration at a certain disadvantage as compared with those which receive the less efficient one, in that no food allowance is made for the maintenance of the additional body substance produced. This objection to equalized food intake, however, is pertinent only when the rats of a pair have diverged appreciably in live weight, at which time the difference in the quality of the rations compared has been demonstrated.

Maynard and associates ('32) have discussed this matter of food allowance in the use of the paired-feeding method at some length, and have followed Armsby in making food allowance for the maintenance of the extra gain produced on the more efficient rations, rather than Mitchell, who has modified the method of Armsby, in applying it to nutritional experiments with rats, by feeding the rations to be compared in equal quantities, regardless of difference in gains produced.

In the opinion of the writers the point of view of Armsby and of Maynard and associates is unquestionably correct, and they concede the argument of Maynard and associates that "it would seem theoretically sound in using growth as a measure to endeavor to equalize the food available for this function." Armsby, however, proposed the method for use in experiments with cattle, on rations not far from normal, while the present writers have used it in experiments with rats, on rations some of which are so far from normal as to result in physiological conditions distinctly different from those which Armsby had in mind; and the writers feel that

it would be more advisable to feed equal quantities of the rations to be compared, even though this procedure should submit the animals on the more efficient rations to a certain technical disadvantage, than to attempt exactly to equalize the quantities of food supplied in excess of the maintenance requirement, for the reason that there is no established basis of computation of the maintenance requirements of growing rats, on either normal or deficient diets.

Also, it seems questionable whether the quantity of food which just prevents a young rat from growing could be considered to be the quantity which it uses for maintenance when on full feed.

Further, Lusk ('28) has repeatedly pointed out that undernutrition may markedly diminish the basal metabolism (which constitutes the larger part of the maintenance requirement); and it is quite conceivable that a rat on a restricted food intake may use a part of its food energy for growth which could be considered as used for maintenance when the animal is on full feed; though, strictly speaking, maintenance requirements can be measured only on a maintenance plane of nutrition, and the significance of such measurements with reference to other planes of nutrition must rest upon assumption alone.

Again, growth may be diminished by deficiency of a single essential nutrient, though the energy intake exceeds the maintenance requirement; and it is by no means certain, in advance of experimental determination, whether the effect of a given nutritive deficiency will be to increase or to decrease the maintenance requirement.

In giving both animals of a matched pair the same quantity of food (in a comparison of diets differing in nutritive value), regardless of difference in gain in weight, it is true that as they diverge in weight, in response to the differences in the rations, the smaller animal will come to receive more food in relation to its body weight than will the larger animal, which receives the more efficient ration; and, at first thought, it would appear that the larger animal comes to be at a comparative disadvantage on this account; but it is necessary to

bear in mind the fact that the nutritive requirements of the animals, on the two treatments, differ reversely as the efficiency of the rations, and that whatever the theoretical inequity experienced by the larger animal it has become the larger because it is actually the more liberally fed of the two.

If the metabolizable energy of the two diets is essentially unaffected by the difference in nutritive value—as is likely to be the case—the difference in the gain of the two animals may be considered as an expression of the fact that with the more deficient diet the sum of the maintenance requirement of energy and the heat increment (that is, the heat loss) is greater than with the more efficient diet.

It seems not to be possible to compare two diets on a perfectly equitable quantitative basis unless both are nutritively complete and perfect.

The method of the present experiment, obviously, affords no basis for separation of the maintenance requirement of energy and the heat increment (specific dynamic effect). These two items will therefore be considered together, as the heat production or the heat loss. It may be well to observe, however, that the maintenance requirement of energy is a practical measure which in order to be significant in relation to the utilization of a diet for body increase, as in the present investigation, or in other practical relations, must be determined under conditions as nearly as possible those to which it is to apply. It is affected not only by the weight of the animal but also by its physiological condition—by its nutritive reserves—and is unaffected by the diet only as it may be assumed that it remains unchanged while the entire increase in heat production accompanying the use of food is considered to be energy expense of food utilization.

It is the belief of the writers, therefore, that the maintenance requirements of growing rats cannot be computed, on the basis of present understanding, with such accuracy as would make it proper to employ the results as a basis for food assignment in critical nutritional research.

The general method of paired feeding, however, is especially valuable in critical nutritive comparisons in that it yields results which can properly be subjected to unbiased statistical evaluation.

THE EXPERIMENTAL DIETS

The cystine-deficient diet was made up as follows: skim milk powder, 23.50 per cent; Osborne and Mendel salt mixture, 4.00 per cent; butter fat, 15.00 per cent; sucrose, 10.00 per cent; cellu flour, 4.00 per cent; NaCl, 1.00 per cent; starch, 41.50 per cent; and irradiated yeast, 1.00 per cent.

The cystine-supplemented diet contained 0.24 per cent added l-cystine, obtained from the Eastman Kodak Co., the skim milk powder being diminished to 22.99 per cent, and the starch increased to 41.77 per cent. The food was weighed out daily with a chemical balance, and a record kept of the quantities of food refused.

In an experiment concerned with cystine deficiency it is of interest to know the approximate cystine content of the rations, and in the present study the cystine of the unsupplemented diet was contained in skim milk powder and yeast.

Vickery and White ('33) recently presented results from which it is assumed that casein contains 0.22 per cent, and lactalbumin 2.56 per cent of cystine.

Assuming that milk contains 2.95 per cent casein, 0.52 per cent lactalbumin, and 0.05 per cent globulin (L. A. Rogers, associates of, '28), the protein of milk may be divided as follows: casein, 83.81 per cent; lactalbumin, 14.77 per cent; globulin, 1.42 per cent.

The cystine-deficient ration contained, by analysis, 8.54 per cent protein. The cystine content of the yeast used in the ration may be considered to be about 0.270 per cent.[2] The yeast constituted 1 per cent of the ration, and thus furnished about 0.524 per cent of protein and 0.003 per cent cystine in the ration.

[2] According to a private communication from F. A. Csonka.

On the basis of the data given the cystine contributed to the diet by the milk powder was 0.045 per cent, which value was increased by the yeast to a total of 0.048 per cent cystine in the entire diet.

In consideration of their yeast content alone the diets were deficient in vitamins B and G, though the skim milk powder doubtless contained some of these vitamins. During the fifth week of the experiment, the appearance of rats nos. 25 to 28, inclusive, indicated a possible vitamin B deficiency. One gram of yeast per week was therefore given to all rats during the sixth and seventh weeks, but was then discontinued because the rats on the cystine-deficient diet gained weight as rapidly as did their pair mates. A weekly feed consumption of 45 gm. signifies a cystine intake of about 0.022 gm. The above additional gram of yeast per week, which equalized the growth of pair mates, raised the cystine intake of the basal diet by about 12 per cent.

Although it has been known that yeast is rich in amino acids, including cystine, it has been frequently used as a source of vitamins, in studies of amino acid deficiencies (Jackson and Block, '32; Mitchell and Beadles, '30; Mitchell and Carman, '26; Rose, '31).

After the completion of the present experiment an article by du Vigneaud et al. ('32) appeared in which the yeast in a low-cystine diet was advantageously replaced by a milk vitamin concentrate.

EXPERIMENTAL CAGES

The rats were kept in individual cages (fig. 1), so equipped that very satisfactory separate collection of feces and of urine was accomplished. The main part of the cage fitted into an 8-inch crystallizing dish and was supported with its lower edge about 1½ inches below the edge of the dish by means of three wires soldered to the cage in such manner as to support it from the edge of the dish. The floor of the cage, about ¾ inch below the edge of the dish, was made of ½-inch mesh galvanized screen, and about an inch below the floor of the

cage an 8-mesh stainless steel wire screen served to collect the feces, and to allow the urine to drop through into the dish.

The top of this cage is closed when in use, by an inverted pie pan.

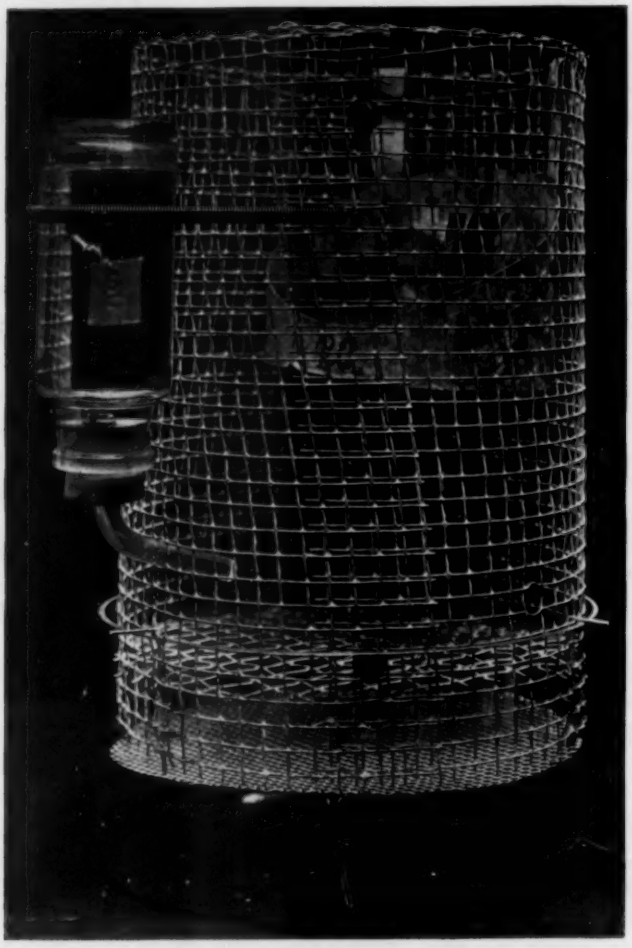

Fig. 1 A metabolism cage for rats.

The feeders, as shown in the figure, were constructed of galvanized iron, and were suspended from the upper edge of the cage by strips of the same material as that of which they were made.

A 50-cc. beaker was used as the feed container, this being held in place in the middle of the feeder by a close-fitting galvanized iron cup soldered to the floor of the feeder. The beaker was held in this galvanized iron cup by strips of sheet copper soldered to the edge of the cup and bent down over the edge of the beaker.

In order to prevent the rat from scooping the food out of the beaker a loose ring of galvanized iron, across which were soldered two wires at right angles to each other, was placed on top of the food. This device accomplished the intended purpose and should be valuable with unpalatable diets, but in the present experiment was found to be usually unnecessary.

This feeder was virtually perfect in preventing the scattering of food.

DAILY ROUTINE

The feed cups were removed from the cages each day at 8 A.M. and replaced at 4 P.M. This interval without food caused the rats to eat more eagerly, and it also helped, at the start of the experiment, to prevent the animals from forming the habit of depositing feces or urine in the feed cups. The feces were removed from the screens daily and kept in loosely stoppered bottles at room temperature.

The dishes and screens were washed with boiling water daily; the individual washings were made up to volume (250 cc.), and the solution was filtered and sampled for analysis, two aliquots being taken, one (50 cc.) for nitrogen and one (100 cc.) for carbon and energy determinations.

The aliquot for nitrogen determination was put into a 2½-liter bottle, with 15 cc. concentrated H_2SO_4 as a preservative, the nitrogen being determined on the accumulated aliquots when the bottle had become nearly full.

No preservative was used in the crystallizing dishes. Any loss of nitrogen occurring during the intervals between the daily washings proved to be negligible, as shown by the satisfactory recovery of feed nitrogen in the feces, urine, and body gain, which averaged 98.4 per cent for the twenty rats, with a coefficient of variation of only $\pm$ 1.43. The aliquots for energy and carbon determination were placed in pyrex dishes and dried in an oven, equipped with a fan, at a temperature of 50°C. One such composite was thus accumulated for each rat.

FINAL OBSERVATIONS

At the end of the experiment, energy, carbon, and nitrogen were determined in the dried urines. Losses of carbon and energy occurring during the process of drying were computed from the loss of nitrogen, on the basis of the assumption that the relationship between the energy and the carbon lost was as in urea. The percentages of carbon and energy lost during drying were found to average 4.2 and 5.7, respectively. The determined carbon and energy values of the urines were corrected for these losses.

At the end of 14 weeks the rats were killed by means of gas; the contents of the alimentary tract were removed and discarded; and the bodies were dried in vacuum desiccators. They were then exposed to room air for several days, weighed, cut up with heavy shears, and extracted with ether for 48 hours. The residue was again exposed to room air for several days, weighed, ground in a hand food chopper, until satisfactorily fine, and then subjected to analysis.

Energy determinations were made, on both the ether extract and the extraction residue, by means of the bomb calorimeter, the details of procedure being as described by Fries ('12).

This accounting for the energy of the entire bodies of the rats, in two samples, furnished an unusually accurate basis for measuring food utilization. Energy determinations were made in duplicate and were accepted only when the difference between repeats was less than 1 per cent.

DISCUSSION OF RESULTS

In the course of this experiment there were 289 refusals of feed, 216 being by the cystine-deficient rats. This deviation of 71.5 from the mean, if chance only determined the result, is 8.41 times the standard deviation (8.50), and would occur by chance once only in millions of trials. Thus, the cystine-deficient rat was definitely the restricting member of the pair, with respect to food intake. This finding contrasts with that of Beadles et al. ('30) who found no significant difference in the number of feed refusals among cystine-deficient rats as compared with cystine-supplemented rats.

All the rats which received the cystine-supplemented ration made greater growth than did their pair mates, though in the case of pair no. 9 the difference was very small (chart 1). Among 140 comparisons of weekly gains, the cystine rat was favored 104.5 times, cases of equal gains being equally divided. The standard deviation of 140 events which may occur with equal probability in either of two ways is 5.92. The deviation of 34.5 from the expected outcome (70) if neither rat of a pair is favored, is 5.83 times the standard deviation, and would occur by chance once only in many thousands of trials (Fisher, '28). Furthermore, the superiority of the average weekly gain in weight of the cystine-supplemented rats as compared with their pair mates was 2.38 gm., as compared with 1.57 gm. for the few weeks during which the cystine-deficient rats gained more than their mates. The ratio of the gain in body weight to the weight of the dry matter of food eaten was 17.5 per cent for the cystine-deficient rats and 20.4 per cent for their pair mates (table 1).

Throughout the tables of results of this experiment all values represent single animals for 14 weeks, and the calorie values are large calories.

In table 2 it will be observed that in eight cases among ten the cystine-supplemented rat stored more energy than its pair mate, the average difference being 10.8 per cent of the smaller gain. A statistical study by 'Student's' method ('Student,' '08), reveals odds of 55 to 1 that the cystine-sup-

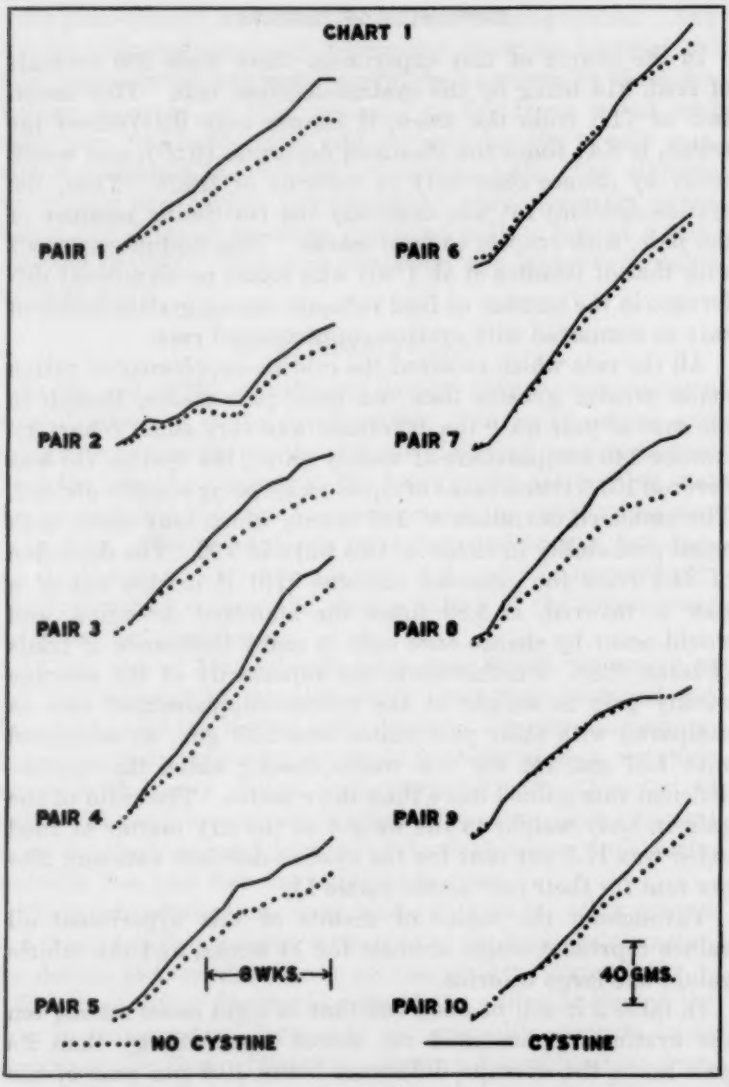

Chart 1 The growth of albino rats, during 14 weeks, as affected by cystine deficiency.

TABLE 1

Gain in body weight[1] related to dry matter of feed

PAIR NO.	FOOD EATEN (DRY MATTER)	CYSTINE DEFICIENT DIET		CYSTINE SUPPLEMENTED DIET	
		Gain in body weight	Ratio of gain to D. M. of food	Gain in body weight	Ratio of gain to D. M. of food
	gm.	*gm.*	*per cent*	*gm.*	*per cent*
1	578.7	80.78	14.0	104.03	18.0
2	488.8	63.39	13.0	75.76	15.5
3	563.7	88.62	15.7	113.25	20.1
4	715.8	152.02	21.2	169.45	23.7
5	544.8	93.59	17.2	110.92	20.4
6	724.1	133.18	18.4	152.50	21.1
7	707.2	140.97	19.9	146.87	20.8
8	632.7	103.35	16.3	135.78	21.5
9	505.0	93.00	18.4	93.79	18.6
10	578.1	117.96	20.4	138.35	23.9
Average	603.9	106.69	17.5	124.07	20.4

[1] Excluding contents of alimentary tract.

TABLE 2

Energy of body gain related to gross energy, and to metabolizable energy of feed

PAIR NO.	FEED ENERGY	CYSTINE DEFICIENT DIET				CYSTINE SUPPLEMENTED DIET				
		Body gain		Metabolizable energy[1]	Body gain as percentage of metabolizable energy	Body gain		Metabolizable energy[1]	Body gain as percentage of metabolizable energy	Body gain of energy of 'no cystine' rat as percentage of gain of pair mate
			Per cent of feed energy				Per cent of feed energy			
	Cal.	*Cal.*		*Cal.*	*per cent*	*Cal.*		*Cal.*	*per cent*	*per cent*
1	2770	240.4	8.7	2329.9	10.3	333.8	12.1	2400.0	13.9	72.0
2	2340	234.3	10.0	2000.0	11.7	255.1	10.9	2019.6	12.6	91.8
3	2698	319.2	11.8	2371.1	13.5	406.4	15.1	2394.4	17.0	78.5
4	3427	596.9	17.4	2988.6	20.0	586.4	17.1	2968.6	19.8	101.8
5	2608	367.9	14.1	2281.1	16.1	375.5	14.4	2280.6	16.5	98.0
6	3467	493.1	14.2	3012.1	16.4	552.8	15.9	3024.5	18.2	89.2
7	3386	431.6	12.7	2987.5	14.4	462.8	13.7	2969.6	15.6	93.3
8	3029	333.7	11.0	2615.0	12.8	478.5	15.8	2651.5	18.0	69.7
9	2418	369.4	15.3	2131.1	17.3	265.5	11.0	2114.2	12.6	139.1
10	2768	352.6	12.7	2424.4	14.5	426.3	15.4	2432.3	17.5	82.7
Average	2891	373.9	12.8	2514.1	14.7	414.3	14.1	2525.5	16.2	91.6

[1] Uncorrected for non-metabolizable energy of protein gained.

plemented diet produced greater energy gains than the cystine-deficient diet.

In two cases, however (pairs nos. 4 and 9) the cystine-deficient rats stored more fat and more energy than did their pair mates (table 4). These two rats also made more efficient utilization of metabolizable energy for body gain, thus showing that gain in live weight may not accurately indicate the efficiency of utilization of feed energy.

A smaller quantity of cystine in the deficient ration might conceivably have reversed the result with pair no. 4, but in the case of pair no. 9 it must be concluded that the cystine-deficient rat was definitely superior to its pair mate in its inherent capacity to synthesize and to store fat. It is perhaps significant in this relation that the rat of pair no. 9 which received the cystine-supplemented diet was the smallest individual among the twenty used in the experiment. The small initial weight of the rat (31 gm.), considered together with the inferiority of its energy gain on the cystine-supplemented diet, suggests that this individual was physiologically inferior to its mate.

The metabolizable energy of the two rations was, on an average, essentially alike (table 2).

The energy of the urine averaged 63.4 Cal. for the cystine-deficient rats, and 60.6 Cal. for the cystine-supplemented rats, this difference being 4.62 per cent of the smaller quantity. Although in nine cases out of ten the energy loss in the urine was greater with the cystine-deficient rat than with the pair mate, the odds are only 18 to 1 that the cystine-deficient diet was responsible for the greater loss of energy in the urine. In the case of pair no. 4 the energy of the urine of the cystine-deficient rat was 11.6 Cal. lower than that of its pair mate.

The energy of the feces (table 5), which is also involved in the measurement of metabolizable energy, was, on an average, 8.6 Cal. higher for the cystine-deficient rats, but with odds of only 8 to 1 that this finding did not occur by chance. This energy loss in feces, therefore, was 2.74 per cent less with the rats which received the cystine-supplemented diet.

The heat production (table 3), including the maintenance requirement and the heat increment due to the food, was obtained by subtracting from the gross energy of the food that of the feces, the urine, and the body gain. Although the differences were small, the greater heat loss occurred, in nine cases among the ten, with the cystine-deficient rat, the exception being, again, with pair no. 9. The average difference in heat production was 1.35 per cent less with the rats which received the cystine-supplemented diet, the significance of this difference being expressed by odds of 38 to 1.

TABLE 3

Heat loss related to energy of feed

PAIR NO.	FEED ENERGY	CYSTINE DEFICIENT DIET		CYSTINE SUPPLEMENTED DIET	
		Heat loss		Heat loss	
			Per cent of feed energy		Per cent of feed energy
	Cal.	*Cal.*		*Cal.*	
1	2770	2089.5	75.4	2066.2	74.6
2	2340	1765.7	75.5	1764.5	75.4
3	2698	2051.9	76.1	1988.0	73.7
4	3427	2391.7	69.8	2382.2	69.5
5	2608	1913.2	73.4	1905.1	73.0
6	3467	2519.0	72.7	2471.7	71.3
7	3386	2555.9	75.5	2506.8	74.0
8	3029	2281.3	75.3	2173.0	71.7
9	2418	1761.7	72.9	1848.7	76.5
10	2768	2071.8	74.8	2006.0	72.5
Average	2891	2140.2	74.1	2111.2	73.2

The 40.4 Cal. (10.8 per cent) difference in energy gain, in favor of the rats which received the cystine-supplemented diet, comprised 29 Cal. difference in heat, 8.6 Cal. in feces and 2.8 Cal. in urine. The difference in heat may be allocated, by definition, either to the heat increment due to the food or to the maintenance quota.

Thus, the important difference in the energy value of the rations was not digestibility, nor in metabolizability, but in the proportion of the metabolizable energy which was utilized for body gain.

Table 2 shows the energy of the body gain as percentage of the metabolizable energy, the energy gain of the rats on the deficient diet being 91.6 per cent as much as that of their pair mates on the cystine-supplemented diet. If pair no. 9

TABLE 4

Nitrogen of body gain related to fat and energy of body gain and to nitrogen of feed

PAIR NO.	NITROGEN OF BODY GAIN	FAT GAINED		ENERGY GAINED			NITROGEN OF FEED	
			Per gram nitrogen gained		As protein	As fat		Utilized for body gain
Cystine deficient diet								
	gm.	*gm.*	*gm.*	*Cal.*	*per cent*	*per cent*	*gm.*	*per cent*
1	2.56	15.4	6.0	240.4	39.1	60.9	8.55	29.9
2	1.88	17.5	9.3	234.3	29.3	70.7	7.24	26.0
3	2.57	23.7	9.2	319.2	30.3	69.7	8.33	30.9
4	3.97	48.9	12.3	596.9	23.1	76.9	10.54	37.7
5	2.42	30.1	12.4	367.9	22.8	77.2	8.02	30.2
6	3.74	38.4	10.3	493.1	26.6	73.4	10.63	35.2
7	4.23	30.0	7.1	431.6	35.0	65.0	10.38	40.8
8	3.13	23.9	7.6	333.7	33.1	66.9	9.30	33.7
9	2.60	29.7	11.4	369.4	24.9	75.1	7.45	34.9
10	3.45	25.7	7.4	352.6	33.2	66.8	8.51	40.5
Average	3.06	28.3	9.3	373.9	29.7	70.3	8.90	34.0
Cystine supplemented diet								
1	3.48	22.6	6.5	333.8	35.9	64.1	8.55	40.7
2	2.30	18.0	7.8	255.1	32.9	67.1	7.24	31.8
3	3.37	30.3	9.0	406.4	29.7	70.3	8.33	40.5
4	4.93	44.3	9.0	586.4	28.8	71.2	10.54	46.8
5	3.40	27.8	8.2	375.5	30.5	69.5	8.02	42.4
6	4.45	42.9	9.6	552.8	27.2	72.8	10.63	41.9
7	4.60	32.6	7.1	462.8	34.4	65.6	10.38	44.3
8	4.01	35.9	9.0	478.5	29.3	70.7	9.30	43.1
9	3.17	16.7	5.3	265.5	42.1	57.9	7.45	42.6
10	4.16	29.8	7.2	426.3	34.7	65.3	8.51	48.9
Average	3.79	30.1	7.9	414.3	32.6	67.4	8.90	42.3

is omitted the average energy gain from the deficient diet is diminished to 86.3 per cent of that from the cystine-supplemented diet.

It may be seen from table 4 that in every case the cystine-supplemented rat synthesized more body protein than its

pair mate, the average difference being 24 per cent; and, likewise, without exception, eliminated less nitrogen in the urine. No consistent difference was found in nitrogen elimination through the feces.

The ratio of carbon to nitrogen in the urine, as explained by Bickel and Kauffman ('25), is considered to have a definite significance in the metabolism of human beings. They found the ratio to increase in cases of avitaminosis, due to an increased excretion of carbon compounds in the urine.

Bickel and Remesow ('27) noted the influence of the composition, especially the amino acid content, of food, on the C to N ratio in the urine. They found that when certain food protein was in part replaced by an amino acid, additional nitrogen was retained without there being increased oxidation of protein, which resulted in a higher C to N ratio in the urine.

It will be seen in table 6 that while the C:N ratios of pair mates do not differ significantly, the ratios are all very high, ranging from 1.6 to 2.2, the average for the rats receiving the cystine-supplemented diet being 2.0, and for their pair mates 1.8.

TABLE 5

Digestibility of protein and energy producing nutriment

PAIR NO.	FEED NITROGEN	FEED ENERGY	CYSTINE DEFICIENT DIET				CYSTINE SUPPLEMENTED DIET			
			Nitrogen		Energy		Nitrogen		Energy	
			Feces	Digested	Feces	Digested	Feces	Digested	Feces	Digested
	gm.	*Cal.*	*gm.*	*per cent*	*Cal.*	*per cent*	*gm.*	*per cent*	*Cal.*	*per cent*
1	8.55	2770	2.04	76.1	366.5	86.8	1.85	78.4	307.9	88.9
2	7.24	2340	1.66	77.1	282.4	87.9	1.72	76.2	270.2	88.5
3	8.33	2698	1.77	78.8	267.2	90.1	1.48	82.2	244.8	90.9
4	10.54	3427	2.38	77.4	381.5	88.9	2.00	81.0	389.9	88.6
5	8.02	2608	1.51	81.2	268.1	89.7	1.63	79.7	275.2	89.4
6	10.63	3467	2.24	78.9	370.0	89.3	1.98	81.4	360.0	89.6
7	10.38	3386	2.06	80.2	326.8	90.3	2.05	80.3	345.0	89.8
8	9.30	3029	1.95	79.0	352.8	88.4	1.77	81.0	316.4	89.6
9	7.45	2418	1.37	81.6	233.5	90.3	1.48	80.1	254.5	89.5
10	8.51	2768	1.68	80.3	286.9	89.6	1.59	81.3	285.7	89.7
Average	8.90	2891	1.87	79.1	313.6	89.1	1.76	80.2	305.0	89.5

It is shown in table 4 that the cystine-supplemented rats, on an average, utilized 42.3 per cent of the food nitrogen for body gain, while the cystine-deficient rats utilized 34.0 per cent of their food nitrogen. In an unpublished experiment of this series, in which 17.7 per cent of protein was supplied by yellow corn and casein, there being no known nutritive deficiency in the ration, the utilization of food protein for body growth was only 23.7 per cent, the C:N ratio in the urine being 0.9. In a third experiment of this same series,

TABLE 6

Urinary nitrogen related to urinary carbon

PAIR NO.	CYSTINE DEFICIENT DIET			CYSTINE SUPPLEMENTED DIET		
	Urinary carbon	Urinary nitrogen	Carbon-nitrogen ratio	Urinary carbon	Urinary nitrogen	Carbon-nitrogen ratio
	gm.	*gm.*		*gm.*	*gm.*	
1	7.75	3.79	2.0	6.29	3.33	1.9
2	5.95	3.70	1.6	5.12	3.28	1.6
3	6.01	3.63	1.7	5.90	3.27	1.8
4	8.00	4.19	1.9	7.32	3.46	2.1
5	6.26	3.82	1.6	5.56	2.97	1.9
6	9.54	4.41	2.2	8.74	4.05	2.2
7	7.29	3.76	1.9	7.41	3.44	2.2
8	6.32	4.06	1.6	6.57	3.37	1.9
9	5.62	3.39	1.7	5.27	2.65	2.0
10	6.03	3.22	1.9	5.42	2.50	2.2
Average	6.88	3.80	1.8	6.36	3.23	2.0

in which the complete diet contained 18 per cent protein (casein), 18.2 per cent of the food protein was utilized for growth, with a C:N ratio in the urine of 0.67. The amount of carbon in the urine from the three experiments was not greatly different; and the very high C:N ratios reported in this paper were determined primarily by the low percentage of nitrogen in the rations—about half of that required for optimum growth.

Table 4 also shows that with the rats on the deficient diet 29.7 per cent of the energy of the body gain was represented by protein, and 70.3 per cent by fat, while with the rats on

the cystine-supplemented diet 32.6 per cent of the gain was represented by protein, and 67.4 per cent by fat. In connection with the corresponding difference in utilization of food nitrogen these differences in composition of the body increase are considered significant.

The following statement by Peters and Van Slyke ('31) is pertinent to the finding of the small amounts of urinary nitrogen in this experiment: "The tendency for growth to continue in spite of all obstacles is so great that, provided the caloric value of the food is sufficiently great, the growing child will retain nitrogen if he is given anything in excess of the requirement for endogenous metabolism."

Sugar in the urine was not determined, and such high C to N ratios as were obtained might result from elimination of small quantities of sugar or other non-nitrogenous substance; but the C to N ratio of urea is so low that if the quantity of non-nitrogenous substance in the urine were little affected by the experimental treatment, the very low protein intake which prevailed, and the consequent low urea outgo, alone, would contribute prominently to the production of high C to N ratios. An indication that the high ratios of carbon to nitrogen found in this experiment result from the low protein intake is the finding of Forbes and associates ('33), who have reported ratios as high as 4.0, the values varying inversely as the amount of protein in the rations, none of which was known to be pathological.

SUMMARY

Twenty young growing rats were fed for 14 weeks, by the paired-feeding method, to demonstrate the ways in which cystine affects the utilization of food nitrogen and energy when added to a cystine-deficient diet.

The diets compared contained 8 per cent of protein, from skim milk powder; one diet was supplemented with 0.24 per cent of l-cystine.

Cystine deficiency unfavorably affected the appetite, but was without certain effect on the digestibility of the food protein.

The rats which received the additional 0.24 per cent of cystine made the greater growth, and stored 10.8 per cent more energy and 24.4 per cent more nitrogen than did the controls.

During the 14 weeks, the rats which received the cystine stored on the average 40.4 Cal. more than did the controls. This gain was accompanied by 29 Cal. less loss in heat, 8.6 Cal. less loss in feces, and 2.8 Cal. less loss in urine, by the rats which received the cystine supplement.

These differences in energy loss, when related to the gross energy of the diets, were 0.1 per cent as urine, 0.3 per cent as feces, and 1.0 per cent as heat.

The ratio of carbon to nitrogen in the urine was approximately 2.0, and did not differ significantly between the two groups. The unusual magnitude of this value was due to the low proportion of protein in the diet.

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DISAPPEARANCE OF VITAMIN C FROM THE ADRENALS OF SCORBUTIC GUINEA PIGS

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(Received for publication November 11, 1933)

Szent-Gyorgyi ('28) has shown that the cortex of normal ox adrenals contains a substance which reduces a neutral 0.4 per cent silver nitrate solution. He identified it as being an hexuronic acid. Svirbely and Szent-Gyorgyi ('33) found that the hexuronic acid has marked antiscorbutic properties. They gave it the name of 'ascorbic acid.' King and Waugh ('32) have shown that crystalline vitamin C from lemon juice is identical with the 'hexuronic acid' Szent-Gyorgyi isolated from adrenals. We have previously reported ('33) our observation that the cortex of adrenals from guinea pigs having scurvy does not reduce 0.4 per cent silver nitrate solution.[2] Moore and Ray ('32), Svirbely and Szent-Gyorgyi ('33), and Harris and Ray ('33) have reported similar observations.

We have used Szent-Gyorgyi's silver staining reaction as a means of following the rate of disappearance of ascorbic acid from the adrenals during the onset of scurvy. Szent-Gyorgyi ('28) found that ascorbic acid from adrenals reduces iodine quantitatively. We attempted to adapt this method for determining the amount of ascorbic acid in the adrenals and to follow its rate of disappearance during the onset of scurvy. Since iodine reacts with other substances known to

[1] This article is taken from a dissertation presented by Arthur E. Siehrs to the Graduate School of Northwestern University, in partial fulfillment of the requirements for the degree of doctor of philosophy.

[2] Preliminary report presented before the Society for Experimental Biology and Medicine, Chicago, May, 1932.

be present in the adrenal, the method is not specific for ascorbic acid. The method determines the quantity of iodine reducing substances present. Harris and Ray ('32) have found that an iodimetric method for determining the ascorbic acid content of lemon and orange juice is unsatisfactory, since iodine reacts with other substances present in the fruit juices.

EXPERIMENTAL

Rate of disappearance of ascorbic acid as indicated by silver staining reaction. Guinea pigs weighing 150 to 160 gm. were fed oats and carrots for 8 days. On the eighth day, two were killed. The adrenals were removed. One from each animal was sliced and placed in a neutral 0.4 per cent silver nitrate solution for 15 minutes. The pieces were exposed to the light from a 115 watt Mazda bulb at a distance of 8 inches. Both the cortex and the medulla reduced the silver nitrate solution; however, the medulla reduced more slowly than did the cortex. The cut surfaces of the cortex of adrenals from normal guinea pigs turn very dark brown, almost black. Szent-Gyorgyi ('28) states that the medulla does not reduce neutral silver nitrate.

The remaining animals were placed on the scorbutic diet of Sherman, LaMer and Campbell ('22). The animals were killed at various intervals from the second to the twenty-second day of the diet period, and the reducing power of their adrenals was determined similarly. The data are given in table 1.

There is a noticeable decrease in the intensity of reduction in the cortex on the second day of the diet period. The intensity of reduction decreased progressively up to the sixth day. The color of the cut surface changes progressively from a very dark brown, almost black, to a reddish orange on the fifth day. The slight change in appearance on the fifth day may be due to the presence of a very small amount of ascorbic acid or some other reducing substance; or it may be due to the precipitation of the proteins by silver nitrate. In the table we refer to this as 1+ reduction. Szent-Gyorgyi ('28)

states that adrenin does not reduce neutral silver nitrate. After the sixth day, if any reduction takes place in the cortex, the extent is so small that it is scarcely evident. Also, after the sixth day, it was noticed that the capsule of the adrenals showed a more marked change in appearance than did the cut surface of the cortex. The animals did not begin to lose weight until after the ninth day. Autopsies showed no hemorrhagic areas about the joints and sternum, with but one exception. Beginning on the eighteenth day, two animals were

TABLE 1

NO. OF ANIMAL	DAYS OF SCORBUTIC DIET	INITIAL WEIGHT	WEIGHT CHANGE	EXTENT OF REDUCTION	
				Cortex	Medulla
		gm.	gm.		
1	0	216	+ 4	++++	++++
2	1	208	+ 12	+++	+++
3	2	170	+ 8	++	+++
4	3	160	+ 8	+	++
5	6	225	+ 7	++	+++
6	8	158	— 16	+	+++
7	10	180	+ 1	+	+++
8	16	172	— 29	+	++
9	18	236	— 16	+	+++
10	18, then fed 3 cc. orange juice for 2 days	192	— 24	+++++	++++
11	18, then fed 3 cc. orange juice for 4 days	182	— 10	+++++	++++

fed 3 cc. of orange juice daily by dropper. The animals were killed on the twentieth and twenty-second days, respectively, and their adrenals examined for reducing power toward silver nitrate. The sliced adrenals turned black in the silver nitrate solution. The intensity of reduction after orange juice feeding was greater than the reduction in the case of the guinea pigs which had been fed a normal diet. This shows that the cortex of the adrenals is still normal as it relates to the metabolism of ascorbic acid and that scurvy is not a condition in which the cortex is rendered permeable to ascorbic acid, permitting its loss from the body.

Five adult guinea pigs, weighing 380 to 420 gm. were fed a diet of oats and carrots for 2 weeks. They were killed and their adrenals were removed; one from each was sliced and placed in the silver nitrate solution according to the usual method. The cortex, medulla and capsule turned black. Adrenals from guinea pigs weighing 150 to 190 gm. under similar conditions gave a very dark brown but not a definite black. This indicates that the ascorbic acid content of the gland varies with the age of the animal.

Rate of disappearance of reducing substances in adrenals as indicated by an iodimetric method. Szent-Gyorgyi ('28) showed that ascorbic acid reacts quantitatively with iodine. An attempt was made to adapt this method to the determination of ascorbic acid in adrenals. Gluthathione, cysteine and thioneine react with iodine under the conditions of the analysis. We found that adrenin does not react with iodine under these conditions. Tunnicliffe ('25) has reported that iodine does not react with urea, uric acid, creatinine, glucose and fructose. Hence, the iodimetric method determines the amount of iodine reducing substances in the adrenals and not ascorbic acid alone.

Twelve guinea pigs, weighing 350 to 500 gm., were kept together in a large cage and fed a normal diet of oats and carrots supplemented with green vegetables. At varying time intervals during a 36-day period, one animal was removed to another cage and placed on the scorbutic diet. The length of time intervals was such that at the end of the experimental period, one animal had been on the scorbutic diet for 36 days, one for 1 day, and the other ten for periods varying from 1 to 36 days. Two within the same weight range were continued on a normal diet. These animals had been used in a previous study but had been allowed to recover. It has been shown that adult guinea pigs of this size and age are much more resistant to scurvy than young guinea pigs weighing between 150 and 250 gm. The left adrenal was always used for the determination. It was triturated with 10 cc. of 10 per cent trichloracetic acid, filtered, washed, and made up to 50 cc. in

a volumetric flask. Twenty cubic centimeters of this extract was placed in an Erlenmeyer flask. Four cubic centimeters of 25 per cent potassium iodide, 1 cc. of concentrated hydrochloric acid, 0.5 cc. of 1 per cent starch solution, and a slight excess of 0.002 N iodine solution were added. This was allowed to stand for 3 minutes. The excess iodine was then

TABLE 2

NO. OF ANIMAL	WEIGHT OF ADRENAL	CUBIC CENTIMETERS 0.002 N IODINE SOLUTION	CUBIC CENTIMETERS IODINE PER 0.01 GM. ADRENAL	CUBIC CENTIMETERS IODINE PER 100 GM. BODY WEIGHT	DAYS ON SCORBUTIC DIET	SILVER NITRATE REDUCTION OF ADRENALS		
1	2	3	4	5	6	Cortex	Medulla	
1	0.14	0.78	0.56	0.16	36	+	++	Mild scurvy with slight hemorrhagic joints
2	0.24	0.80	0.32	0.27	32	+	+++	All joints very hemorrhagic, severe scurvy
3	0.19	1.18	0.62	0.30	30	++	+++	All joints hemorrhagic, mild scurvy
4	0.22	1.05	0.48	0.26	27	++	++	All joints very hemorrhagic, severe scurvy
5	0.14	0.90	0.67	0.16	19	+	++	Very slight scurvy
6	0.17	1.12	0.66	0.24	16	++	+++	Very slight scurvy
7	0.16	1.25	0.80	0.22	10	++	+++	Mild scurvy
8	0.36	1.68	0.47	0.34	5	+	++	All joints very hemorrhagic, severe scurvy
9	0.21	0.98	0.47	0.15	3	++	+++	No symptoms of scurvy
10	0.24	1.45	0.60	0.23	1	++	+++	No symptoms of scurvy
11	0.18	1.48	0.82	0.26	0	+++	+++	Normal
12	0.22	1.45	0.68	0.27	0	++++	++++	Normal

titrated with 0.002 N sodium thiosulfate. The blank titration amounted to 0.12 cc. which was subtracted from the difference in titration values. The amount of material available for each determination was insufficient to permit a separate determination of gluthathione. The data are given in table 2. Column 3 shows that there is a progressive decrease in the total iodine reducing substances in the adrenal with the onset of scurvy. Column 4 shows that there is a progressive de-

crease of iodine reducing substances in the adrenal per unit weight of gland after the tenth day. Column 6 shows that there is no significant change in iodine reducing substances in the adrenals compared to body weight throughout the period. The symptoms of scurvy of animals 1 and 8 differ from the usual experience in that animal 8 showed symptoms of scurvy in 5 days whereas animal 1 showed only a mild scurvy after 36 days. The iodine reducing substance in the adrenals of these animals per unit weight of adrenal is in accord with the data found for other animals manifesting comparable severity of symptoms.

DISCUSSION

Heretofore, scurvy has been defined in terms of certain characteristic gross symptoms, such as loss of weight, loosening of teeth, subcutaneous and intramuscular hemorrhages, and soreness and swelling of the joints, which appear, in the case of guinea pigs, after they are fed a scorbutic diet for 2 or 3 weeks. This work indicates that there is prompt and profound decrease in the ascorbic acid content of the body on an ascorbic acid-free diet. As a result of this, there is a profound disturbance of the metabolism of the animal with the development of the characteristic symptoms. The characteristic symptoms are, thus, secondary to the more subtle loss of ascorbic acid in the body. Apparently, ascorbic acid is of primary importance in the metabolism of connective tissue. Scurvy is not due to the loss of ability of the adrenals to store ascorbic acid, for even in severe scurvy the feeding of orange juice is followed by its prompt appearance in the adrenals.

The observation that ascorbic acid promptly disappears from the adrenals on a scorbutic diet and promptly reappears when returned to diet furnishes a basis for a method of assaying the vitamin C content of foods.

SUMMARY

Ascorbic acid in the cortex of the adrenals of guinea pigs progressively disappears during the onset of scurvy. The silver nitrate staining reaction shows that there is a decrease after the animal has been on a scorbutic diet for but 1 day. Minimum staining with silver nitrate is obtained on the sixth day. The feeding of 3 cc. of orange juice to scorbutic animals is followed by the prompt reappearance of ascorbic acid in the cortex of the adrenals. An iodimetric method for the determination of the iodine reducing substances in the adrenals, which includes ascorbic acid, shows that there is a progressive decrease of these substances during the onset of scurvy.

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SPECTRUM ANALYSIS OF MILK ASHES

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(Received for publication January 17, 1934)

Some months ago while spectrum analyses of water from different parts of the Rocky Mountain region were being made, it was noted that some of them contained strong traces of silver. The ashes of human tissues from individuals who had been drinking water containing silver also gave spectrum evidence of strong traces of silver. It was decided to investigate milk for this element and at the same time to note traces of other elements present since considerable attention is now being given to their biological significance.

Milk has been investigated in this way by other workers. Wright and Papish ('29) did not report silver as being present in cow milk and neither did Blumberg and Rask ('33). Zbinden ('31) noted its presence in human milk, but not in cow milk. The samples of milk examined by the first and second named workers were secured in Great Britain and various parts of the United States other than Colorado and whether or not the drinking water or feed the cows were consuming contained silver is not mentioned.

MATERIAL

Samples of milk from twelve cows were examined. These cows were from three different localities, where the drinking water was known to contain silver. Pyrex glass flasks were acid cleaned, rinsed finally with double distilled water, and dried in an inverted position. Into the cleaned flasks was

forced the milk which did not come into contact with the milking hand or with the cleaned teat other than the orifice of the duct. The first two or three streams were not placed in the flasks. Clean paper (not cardboard) covers were then placed over the tops of the flasks and the milk was not allowed to touch the paper. Cork stoppers should not be used as some examined contained silver, some of which was extracted by cold distilled water.

METHOD OF INVESTIGATION

Not to exceed 5 cc. of milk at one time was placed in platinum or silicon dishes, slowly evaporated, and ashed at the lowest possible temperature over a Meeker burner in a room free otherwise from air currents, and after dust had presumably settled. No substance was added to promote the ashing, which occurs best with relatively thin layers of milk. The ash was then covered until used. Controls consisting of powdered graphite from a graphite electrode and a mixture of CaO and MgO, to which the same amounts of double distilled water were added and evaporated after exposure under similar conditions for the same length of time, gave no evidence of contamination by dust.

Samples of the two different drinking waters used by the three herds were evaporated in pyrex dishes over Meeker burners and the residue reserved for spectrum analysis.

Some of the alfalfa grown in the same region and which was being eaten by some of the cows was also ashed, nothing being added to promote the ashing.

The spectra recorded ranged from λ 2300 A. U. to λ 6800 A. U. and were secured by means of a Hilger E 34 medium sized spectrograph. Quartz lenses focused the image of the arc upon the slit of the spectrograph, the images of the incandescent poles not being allowed to fall on the slit.

For the recording of wave lengths lower than λ 5000 A. U. Eastman spectroscopic plates, type III and type IV—O were used. For longer wave lengths, type III—H, for the CaF_2 band, type III—F, and type IV—F were used. Eastman

x-ray developer was used to get maximum density and contrast

A direct current arc was employed for exciting the spectra. Graphite and copper electrodes of 6 mm. diameter were used in establishing the arcs, the lower being made positive to the upper for the passage of a direct current.

Acheson spectroscopic graphite electrodes permit the satisfactory recording of most of the wave lengths. Either 10 or 20 mg. of the substance to be arcked were placed on top of the lower electrode or in a small hole made in the top surface. When 20 mg. of ash were placed in a hole, 13 amperes of direct current were used to heat up the ash promptly so as to liberate the easily volatilized silver and permit the recording of its wave lengths in 4 seconds, the optical system being so diaphragmed that overexposure was avoided. If the ash was placed directly on top of the graphite electrode, 7.5 amperes were used and sufficient time of exposure to the photographic plate given to record satisfactorily the spectrum for the duration of the burn whether 10 or 20 mg. were used, the optical system having been properly adjusted for this. In the latter case, a convenient amount of the ash was placed on top of the lower electrode, the arc established for 2 or 3 seconds, more ash added, the arc reestablished, and thus repeated until the full amount of either 10 or 20 mg. had been arcked.

It was desirable because of the molecular bands produced when graphite electrodes are used that may mask certain wave lengths of some of the elements sought to also use copper electrodes. It was found that 3 to 4 amperes of current were insufficient and 7.5 amperes were used. The ash was placed only on top of the lower positive electrode and arcked in the manner described in the latter part of the last paragraph. The spectra of the substances investigated had placed immediately above them the spectrum of the iron arc by means of a Hartmann diaphragm.

RESULTS

The accompanying table gives the elements found to be present in the milk. The presence and absence of the same elements in the water and in the alfalfa are also recorded together with the inclusion of a few that gave negative or questionable evidence of their presence in the milk.

Trace Elements

	Milk	*Alfalfa*	*Water no. 1*	*Water no. 2*
Al	+	+	+	+
Ba	+	+	+	+
B	+	+	+	+
Cr	+ in seven	+	+	+
Cu	+	+	+	+
F	−	−	+	+
Fe	+	+	+	+
Pb	+	+	+	+
Li	+	+	+	+
Mn	−	+	+	+
Mo	+	+	+	−
Ag	+	+	+	+
Rb	+	+	−	−
Si	?	+	+	+
Sr	+	+	+	+
Sn	+ in a few	+	−	−
Ti	+	+	+	+
V	+	+	+	+
Zn	+	+	+	−

Larger amounts of elements

	Milk	*Alfalfa*	*Water no. 1*	*Water no. 2*
Ca	+	+	+	+
Mg	+	+	+	+
P	+	+	−	−
K	+	+	+	+
Na	+	+	+	+

Traces of silver were shown to be present in the milk from twelve cows drinking water containing silver in traces. Alfalfa fed to the same cows also contained silver. The lines recorded were λ 3280.67 A. U., and λ 3382.88 A. U. The owner of one of the herds stated that a cow drinks 80 or more liters of water and eats at least 9 kg. of dry alfalfa each day.

Molybdenum in traces was present in each milk, in the alfalfa, and in one of the two drinking waters. The wave lengths noted were λ 3798.26 A. U., λ 3864.12 A. U., λ 3902.96 A. U., λ 3132.60 A. U., λ 3170.35 A.U. and λ 3193.98 A.U. Molybdenum was not reported as being present in milk by the investigators mentioned above who used spectrographic methods. H. ter Meulen ('32) using a colorimetric method reported 0.14 to 0.03 mg. of molybdenum per kilogram of milk as well as in the same amounts of blood, bile, eggs, and sundry tissues.

Fluorine was sought in the milk because the drinking waters contained about 2 p.p.m. The CaF_2 band was absent, although the plate recorded the presence of at least 0.02 per cent F. in synthetic test mixtures, confirming a similar former experience to be reported elsewhere. Bone, dentine, and tooth enamel of individuals who had been drinking this fluorine-containing water, contained more fluorine than did the same tissues from individuals who had been living on the Atlantic seacoast (Boissevain and Drea, '33).

Traces of other elements found in the milk and already reported upon by other investigators are aluminum, barium, boron, chromium, copper, iron, lead, lithium, rubidium, strontium, titanium, vanadium, and zinc. Tin was found in three or four of the milks and is questionably present. The presence or absence of silicon is questionable.

Manganese was absent from the milk, although strong traces were found in one of the waters as well as in the alfalfa, and weak traces in the other water.

Calcium, magnesium, phosphorus, potassium, and sodium were present in the usual large amounts.

SUMMARY

Silver and molybdenum are added to the list of 'trace' elements in cow milk as demonstrated by spectrographic methods.

Silver was found in the milk of each of twelve cows drinking water and eating alfalfa containing strong traces of the

same element. Molybdenum was present in the milk of twelve cows. Either the alfalfa, the drinking water, or both contained molybdenum.

Manganese, although present in the water and in the alfalfa, was not demonstrated to be present in the milk.

Fluorine, which was present in the water to the amount of 2 p.p.m. was not shown to be present, although the test synthetic material recorded F when present in as small concentration as 0.02 per cent.

An account is given of the other elements already reported upon by other investigators.

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AN ADAPTATION OF THE PAIRED-FEEDING METHOD FOR THE DETERMINATION OF THE SUPPLEMENTARY VALUE OF PROTEINS[1]

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(Received for publication January 10, 1934)

The determination of the supplementary value of proteins is at best beset with difficulties. This is particularly true when we are called upon to evaluate the proteins from such diverse materials as those from animal products, from seeds and from the vegetative portions of plants.

During recent years the paired-feeding method (Mitchell and Beadles, '30) has found considerable application in the determination of the amino acid deficiencies of proteins. In our own laboratory (Haag, '31) we have used this method with a considerable degree of satisfaction in showing that rations in which the proteins are supplied by alfalfa leaf meal are improved by the addition of l-cystine. In addition we have attempted to adapt the paired-feeding idea to a study of the supplementary value between the proteins of alfalfa leaf meal and those of wheat bran.

Briefly, the method used was as follows: Three rats from the same litter were matched for weight and sex. One member of the triplet was fed a ration containing alfalfa leaf meal as the source of protein; the second member was fed a ration containing wheat bran as the source of protein; and the third member was fed a ration consisting of equal parts of the alfalfa leaf meal and wheat bran rations. By trial, the per cent of wheat bran was adjusted to produce approximately

[1] Published as Technical Paper no. 210 with the approval of the director of the Oregon Agricultural Experiment Station.

the same rate of growth as that obtained with the alfalfa ration. An attempt was made to equalize the digestible dry matter, crude fiber, mineral and vitamin contents to an extent which it was hoped would eliminate these factors from further consideration in assessing the nutritive value of the proteins of these rations. The growth obtained on the mixed ration was compared with the average of that obtained on the alfalfa and wheat bran rations. If sufficient care has been exercised in equalizing the experimental conditions referred

TABLE 1

Composition of rations

CONSTITUENTS	NO. 73	NO. 74	NO. 88	NO. 89
	per cent	*per cent*	*per cent*	*per cent*
Alfalfa leaves (1930)	50.0	—	—	—
Alfalfa leaves (1931)	—	—	50.0	—
Wheat bran	—	50.0	—	40.0
Sucrose	10.0	—	10.0	10.0
Dextrin	17.0	22.5	15.5	17.0
Alfalfa stem fiber	—	5.0	—	8.0
$Na\ H_2PO_4 \cdot H_2O$	2.0	—	1.5	—
$CaCO_3$	—	1.5	—	2.0
Nacl	1.0	1.0	1.0	1.0
Yeast	—	—	2.0	2.0
Cod liver oil	1.0	1.0	1.0	1.0
Salad oil	—	—	19.0	19.0
Butterfat	19.0	19.0	—	—
Crude protein	11.0	6.6	11.7	5.3

to above, an increased rate of growth on the mixed ration may, with appropriate reservations, be taken as indicating a supplementary effect between the proteins of alfalfa and of wheat bran.

The alfalfa leaf meal and wheat bran rations were prepared as indicated in table 1. Ration 75 consisted of a mixture of equal parts of rations 73 and 74. Ration 90 consisted of a mixture of equal parts of rations 88 and 89. The digestibility coefficient of the dry matter of these rations was approximately 71 to 72 per cent. The apparent digestibility of the crude proteins of the alfalfa, wheat bran and mixed

rations was 63, 65 and 64 per cent, respectively. The crude fiber content of these rations was approximately equalized by the use of an alfalfa stem fiber preparation from which most of the proteins had been removed by acid-alkali extraction.

The food intake and growth data are summarized in table 2. In the last column the presence or absence of a supplementary effect is indicated by means of a plus or a minus sign, respectively. A supplementary effect is indicated for those triplets where the gain in live weight on the mixed ration was greater than the average of the gains on the alfalfa and wheat bran rations. It will be noted that this method of comparison indicates a supplementary effect in fourteen out of sixteen cases. More significant than the apparently slight supplementary effect between the alfalfa and wheat bran rations is the decided superiority of the proteins of wheat bran over those of alfalfa. This superiority cannot be explained on the basis of greater digestibility.

It is realized that the differences in gain obtained in these experiments are not large, that the energy intake levels are not as high as might be desired, and that the results must be interpreted with appropriate reservations. It does appear, however, that this method of attack promises to be useful in the study of problems of the nature described in this paper.

In conclusion, it may be stated that the results obtained to date (sixteen triplets) indicate that this method of attack promises to prove useful in the study of the nutritive value of the proteins of bulky vegetable and forage crops; that wheat bran proteins are superior to those of alfalfa leaf meal; that this superiority is not due to differences in apparent digestibility; and that there appears to be a slight supplementary effect between the proteins of alfalfa leaf meal and those of wheat bran.

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TABLE 2

Growth and food intake data

TRIPLET NO.	RATION NO.	TYPE OF RATION	INITIAL WEIGHT	FINAL WEIGHT	TOTAL GAIN	TOTAL FOOD	WEEKS	SUPPLEMENTARY EFFECT
			grams	*grams*	*grams*	*grams*		
1	73	Alfalfa	78	125	47	558	10	+
	74	Bran	78	141	63	555		
	75	Mixed	80	136	56	555		
2	73	Alfalfa	85	107	22	507	10	+
	74	Bran	82	127	45	496		
	75	Mixed	82	128	46	509		
3	73	Alfalfa	88	106	18	437	10	+
	74	Bran	82	112	30	449		
	75	Mixed	80	110	30	449		
4	73	Alfalfa	65	93	28	433	10	—
	74	Bran	65	95	30	432		
	75	Mixed	65	93	28	436		
5	73	Alfalfa	73	91	18	468	10	+
	74	Bran	73	100	27	457		
	75	Mixed	73	102	29	469		
6	73	Alfalfa	67	92	25	447	10	+
	74	Bran	67	95	28	451		
	75	Mixed	67	102	35	451		
7	73	Alfalfa	85	83	−2	411	10	+
	74	Bran	85	97	12	419		
	75	Mixed	85	100	15	419		
8	73	Alfalfa	86	92	6	460	10	+
	74	Bran	78	97	19	460		
	75	Mixed	78	102	24	460		
9	88	Alfalfa	90	108	18	270	5	—
	89	Bran	85	97	12	261		
	90	Mixed	85	97	12	263		
10	88	Alfalfa	83	98	15	235	5	+
	89	Bran	80	84	4	262		
	90	Mixed	80	96	16	229		
11	88	Alfalfa	66	86	20	198	4	+
	89	Bran	65	78	13	187		
	90	Mixed	63	86	23	188		
12	88	Alfalfa	63	73	10	152	4	+
	89	Bran	60	67	7	134		
	90	Mixed	60	77	17	153		
13	88	Alfalfa	106	141	35	455	7	+
	89	Bran	106	144	38	455		
	90	Mixed	106	153	47	456		
14	88	Alfalfa	97	133	36	454	7	+
	89	Bran	96	132	36	454		
	90	Mixed	96	143	47	454		
15	88	Alfalfa	92	115	23	393	7	+
	89	Bran	91	112	21	389		
	90	Mixed	93	126	33	393		
16	88	Alfalfa	80	132	52	438	7	+
	89	Bran	77	115	38	431		
	90	Mixed	78	127	49	433		

THE CALCIUM AND PHOSPHORUS CONTENT OF SOME ALABAMA VEGETABLES

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(Received for publication November 18, 1933)

The importance of calcium and phosphorus in the diet is well recognized and considerable attention has also been given to the Ca:P ratio. Comparatively few investigations, however, have been made of the constancy of these elements in foods and considerable use has been made of tables, such as Sherman's ('30), for their calculation in given diets. A few typical studies show that from a nutritional standpoint further work along this line is needed.

A study by Frank and Wang ('25) has shown very significant differences in the calcium content of different samples of certain foods. These samples were procured in the market and the causes of the differences are unknown. Gile and Ageton ('14) showed that while the calcium content of some plants was greatly increased by application of lime to the soil, that of others was not appreciably affected. Storms ('27) found that although New Zealand milk has the same calcium content as that of other countries, the vegetables showed a lower calcium content. The work of Greaves and Hirst ('28) has shown very great differences in the calcium and phosphorus content of certain grains as influenced by variety, soil type and irrigation, and more recently, Greaves and Greaves ('33) have shown that these wheats vary in their nutritive value. Hartwell ('13) found that the percentage of phosphorus in turnips varied with the amount available in the soil, while that of oats and millet was not markedly influenced.

Emmert ('31) and Sewell and Latshaw ('31) found that the phosphorus content of plants was decreased by liming. Cowell ('32) found that the calcium content of the outer leaves of cabbage may be twenty to thirty times that of the inner leaves. Therefore, the extent and significance of these variations should be determined, since diets which would be adequate under certain conditions of plant growth might be deficient under others.

A survey of Alabama shows that the soils of a very considerable part of the state are of very low calcium content and that these soils are not limed in general agricultural practice. Moreover, with a growing tendency to use new kinds of so-called high analysis fertilizers as ammonium phosphate, ammonium nitrate, urea, potassium chloride, etc., the calcium which was formerly added to the soil in fertilizers may be omitted and result in a depletion in the small store of this element. It, therefore, seemed important to determine the calcium content of some vegetable foods grown in these low calcium soils and, because of the relationship of calcium and phosphorus in metabolism, to make a concomitant study of their phosphorus content.

MATERIALS AND METHODS

Although it is recognized that there are other important sources of calcium in the diet and that these need investigation, this study was limited to vegetables. Since leafy vegetables are the chief vegetable source of calcium in the diet and since they are much used in the South, most of the study was confined to them. Some analyses, however, were made of other types of vegetables. The field crops were grown on the Alabama Experiment Station at Auburn, with only one rate of fertilization, 1000 pounds per acre of complete fertilizer. The greenhouse plants were grown at Auburn on five different types of soil, all of which, however, were acid and low in calcium. Plants were grown in each soil which received 0, 250, 500, 1000, and 2000 pounds of superphosphate per acre, respectively.

The sample of edible portion was washed in a small amount of distilled water and was freed of wash water by whirling in cheesecloth and spreading on paper towels. It was then weighed in aluminum pans, dried to constant weight at 105°C., ground to a powder in a ball mill or mortar, redried for 2 hours at 105°C. to remove any adsorbed water and stored in Mason jars. Enough dried sample was weighed in a Sillimanite evaporating dish to give 15 to 40 mg. of calcium, ashed in an electric muffle, weighed for ash and dissolved in hydrochloric acid. The calcium was precipitated as the oxalate and titrated with permanganate.

Enough dried sample to give 1.0 to 4.0 mg. phosphorus was weighed in a Sillimanite evaporating dish and 10 cc. 10 per cent calcium acetate solution were added to prevent loss of phosphorus through volatilization. This was evaporated to dryness on a water bath and ashed in an electric muffle. The ash was covered with water and 10 cc. 6*N* HCl, evaporated to dryness on a water bath to precipitate the silica, and then dissolved in water and 4 cc. 1*N* HCl, in order to give the same pH as the standard. It was next filtered into a 200-cc. volumetric flask, made up to volume and the phosphorus in a suitable aliquot, usually 25 or 50 cc., determined by the Fiske and Subbarow ('25) colorimetric method. It was found that the keeping qualities of the reducing agent for this determination were improved by making the solution up to 500 instead of 400 cc. Five cc. were then used in the determination instead of 4 cc., in order to give the same amount of reducing agent.

RESULTS

The results summarized in table 1 show a wide variation in the calcium and phosphorus content of a given vegetable. This variation was slightly greater on the basis of dry weight than on the basis of the edible portion which is given in table 1. The calcium content of Alabama vegetables is in general low and the phosphorus content high as compared with Sherman's ('30) tables. Lettuce, mustard, and potatoes, however, are low in phosphorus as well as calcium, while the

TABLE 1

Calcium and phosphorus content (percentage of edible portion) of some Alabama vegetables

VARIETY	NUMBER SAMPLES ANALYZED	DRY MATTER			ASH			CALCIUM			PHOSPHORUS			Ca:P		
		Min.	Max.	Ave.	Min.	Max.	Ave.	Min.	Max.	Ave.	Min.	Max.	Ave.	Min.	Max.	Ave.
Beans, lima, dried	13	91.5	96.1	94.4	3.42	3.98	3.70	0.064	0.078	0.071	0.368	0.485	0.406	0.161	0.194	0.173
Cabbage, greenhouse, not headed	43	5.92	20.4	13.7	1.14	2.06	1.55	0.204	0.675	0.380	0.009	0.461	0.024	5.12	61.1	19.8
Cabbage, field, headed	2	8.28	10.1		0.636	0.688		0.030	0.046		0.029	0.040		0.755	1.61	
Chard, field	1		13.1			1.65			0.102			0.068			1.50	
Chinese cabbage, greenhouse, non-heading	26	5.63	10.6	7.25	0.936	1.82	1.27	0.083	0.502	0.226	0.012	0.040	0.022	2.56	28.2	11.7
Chinese cabbage, field, non-heading	18	6.16	11.77	8.25	0.925	1.42	1.16	0.086	0.188	0.121	0.038	0.060	0.050	1.45	4.00	2.54
Collards, field	1		13.3			1.29			0.197			0.073			2.71	
Lettuce, greenhouse	34	3.64	14.4	8.45	0.658	1.49	1.03	0.051	0.097	0.071	0.012	0.040	0.023	1.69	6.15	3.37
Mustard, greenhouse	30	5.55	13.5	8.70	0.990	2.32	1.89	0.156	0.431	0.284	0.014	0.050	0.027	3.76	30.0	12.2
New Zealand spinach, field	3	6.33	10.0		1.50	2.00		0.054	0.090		0.031	0.056		1.15	2.19	
Onion, top, field	2	7.14	9.67		0.661	0.895		0.050	0.064		0.025	0.032		2.02	2.03	
Onion, bulb, field	2	9.92	12.4		0.529	0.665		0.049	0.056		0.040	0.044		1.24	1.28	
Peas, cow, dry	14	85.4	88.2	86.9	2.85	3.78	3.21	0.050	0.080	0.064	0.336	0.503	0.442	0.107	0.169	0.145
Pepper, green, field	1		8.86			0.445			0.010			0.030			0.324	
Potato, Irish, field	2	17.9	22.0		0.970	1.19		0.007	0.008		0.042	0.047		0.167	0.178	
Potato, sweet, field	1		32.5			1.17			0.018			0.039			0.471	
Radish tops, greenhouse	8	6.86	9.08		1.18	1.47		0.170	0.398		0.015	0.034		5.27	23.6	
Tendergreen, green house	22	4.70	8.42	7.10	0.92	1.90	1.32	0.111	0.346	0.217	0.012	0.042	0.025	2.63	19.3	1.00
Tendergreen, field	2	8.86	11.59		1.38	1.39		0.110	0.202		0.056	0.076		1.45	3.60	
Tomato, field	1		5.12			0.467			0.007			0.021			0.341	
Turnip tops, Amer., greenhouse	31	7.05	13.25	9.59	1.27	2.36	1.70	0.212	0.451	0.322	0.016	0.081	0.037	3.23	21.8	10.5
Turnip tops, Amer., field	21	7.58	15.16	10.74	1.16	1.79	1.43	0.180	0.330	0.242	0.046	0.080	0.060	2.39	5.40	4.14
Turnip tops, Jap., greenhouse	20	7.15	10.93	8.53	1.10	1.93	1.47	0.181	0.373	0.274	0.015	0.061	0.031	3.32	20.6	1.01
Turnip tops, Jap., field	9	7.58	14.1	10.20	1.14	1.57	1.31	0.123	0.224	0.172	0.042	0.077	0.060	1.59	4.63	3.12

onion and pepper are high in calcium as compared to Sherman's tables. The amounts found in Alabama vegetables are more comparable to the amounts found in Hawaiian vegetables (Chung and Ripperton, '29) but in general show a slightly higher mineral content. The calcium and phosphorus also show a marked tendency to vary in an inverse manner, thus giving a tremendous variation in the Ca : P ratio.

The greenhouse plants showed a regular increase in phosphorus with increased rates of application of superphosphate.

TABLE 2

Variation of calcium and phosphorus content with soil and superphosphate added

VEGETABLE	SOIL	SUPERPHOSPHATE ADDED, LBS./ACRE	PERCENTAGE OF EDIBLE PORTION				Ca:P
			Dry matter	Ash	Calcium	Phosphorus	
Cabbage	Cecil	250	15.6	1.75	0.487	0.009	55.0
		2000	19.9	1.49	0.358	0.026	14.0
	Eutaw	250	18.0	1.48	0.700	0.011	61.1
		2000	17.4	1.87	0.675	0.030	22.4
	Hartselle	250	17.2	1.34	0.322	0.017	19.2
		2000	18.6	1.80	0.407	0.043	9.56
Lettuce	Cecil	250	9.60	1.27	0.092	0.015	6.15
		2000	11.6	1.16	0.074	0.027	2.74
	Eutaw	250	9.83	0.993	0.067	0.024	2.83
		2000	14.4	1.018	0.081	0.032	2.52
	Hartselle	250	9.67	1.25	0.066	0.020	3.24
		2000	12.1	0.872	0.070	0.038	1.83

The extent of this variation differed with the vegetable and the soil as is illustrated in table 2. The results are shown for three typical soils with two of the rates of superphosphate for two vegetables, cabbage which showed maximum effect and lettuce which showed the least effect of the vegetables studied. In spite of the fact that superphosphate also contains calcium, the variation in the calcium content with increased rates of superphosphate was small and generally showed a decrease until the highest applications were reached where there was a very slight increase.

Japanese varieties of turnips consistently gave a lower calcium content than American varieties grown under the same conditions. Aside from this, the variation with variety or age was not significant when the values were reduced to a basis of edible portion. Older vegetables, of course, had a higher dry weight and hence in terms of dry weight a lower calcium and phosphorus content.

SUMMARY

Analyses were made of a number of Alabama vegetables of which some were grown in the greenhouse on five different soil types with five different rates of superphosphate treatment, and some were grown in the field on one soil type with one fertilizer treatment.

The calcium and phosphorus content of a given vegetable varies widely, usually in opposite directions, so that the Ca : P ratio varies markedly.

Increased rates of superphosphate produce a regular increase in the phosphorus content but a small change in calcium.

ACKNOWLEDGMENT

The author wishes to express appreciation and indebtedness to members of the Horticulture Department for their cooperation in supplying the vegetables which made this investigation possible, and to Mr. J. C. Odom for making many of the analyses reported.

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VARIATION OF BASAL METABOLIC RATE PER UNIT SURFACE AREA WITH AGE

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ONE FIGURE

(Received for publication November 17, 1933)

In a recent article Bruen ('33) gives an excellent summary of the bibliography on this subject. He concludes that the basal metabolic rate during later childhood has been shown in numerous confirmatory investigations to give indications of a definite increase, or an irregularity in its decrease, related to puberty. This variation of the basal metabolism per unit surface area with age during the pubertal period may therefore be analyzed as the mathematical resultant of a fundamental decrease of the basal metabolic rate with age, and a super-imposed cyclic acceleration. This cyclic acceleration of the basal metabolism is not a mere concomitant or effect of the adolescent growth cycle, but represents an independent pubertal metabolic acceleration.

Basal metabolic studies were made on 200 boys whose ages ranged from 9 to 18, the greater number being between 12 and 16 years of age. These boys are all inmates of the New Jersey State Home for Boys in Jamesburg. Our results confirm, in the main, those of DuBois ('16 a, '16 b), Olmstead, Barr and DuBois, ('18), Bierring ('31), Göttche ('26) and Topper and Mulier ('32). Our figures, although they do not show a definite increase, nevertheless do show an irregularity in decrease related to puberty as shown in graph of median trend in chart 1.

The boys, to be tested, were admitted to the institution hospital the evening before. No food was given after the

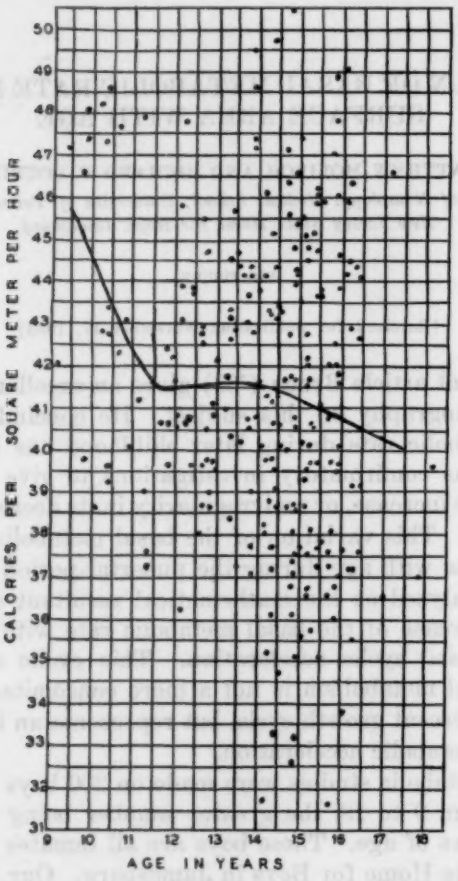

Figure 1

evening meal and nothing to drink after midnight. Successive determinations were made every 24 hours until not more than a 4 per cent difference was obtained. Averages of these check determinations were made. Many cases were excluded be-

cause of inability to obtain a 4 per cent check and because of nervousness. No boys with evident endocrine disturbances were used in the study. All of the tests were done with the Sanborn-Motor-Grafic machine.

Table 1 gives the details of the cases studied. Each age group is separately presented and the averages for each, in

TABLE 1

Calories per square meter of body surface per hour during puberty (males)

AGE	TOTAL CASES	APUBERTAL	PUBESCENT	PUBERTAL	AVERAGE (CALORIES PER SQUARE MILLIMETER)	AVERAGE DEVIATION
9–10	6	4	2		45.9	1.9
10–11	7	6	1		43.1	2.5
11–12	8	7	1		40.1	2.4
12–13	22	11	10	1	41.9	1.9
13–14	29	4	17	8	39.7	3.6
14–15	53	5	20	28	36.2	5.1
15–16	58	1	8	49	41.1	3.0
16–17	14		3	11	41.0	3.5
17–18	3			3	40.9	3.8

TABLE 2

The DuBois normal standard as modified by Boothby and Sandiford (males)
Calories per square meter

AGE[1]	BOOTHBY AND SANDIFORD	AUTHORS DATA
10	49.5	45.0
11	48.6	45.0
12	47.8	42.6
13	47.1	41.4
14	46.2	41.5
15	45.3	40.9
16	44.7	41.9

[1] Age—9 years, 6 months to 10 years, 5 months are included in 10-year group, etc.

Calories per square meter per hour, are given. Our figures are lower than those of Aub and DuBois ('17), and Boothby and Sandiford ('29) (table 2). Correlations were made with the data of Dryer ('20) (observed weight formula) and Benedict and Talbot ('21). Table 3 shows at a glance that between the ages of 12 and 15, the range of the majority of

cases, there appears to be a fairly good correlation between Dreyer's and our data. Too few cases were correlated with Talbot's figures to be of any value.

TABLE 3

Correlations with Dreyer and Talbot (Calories per hour)

AGE	DREYER			TALBOT		
	Number of cases	r	P.E.	Number of cases	r	P.E.
9–10	6	0.81	±0.05	6	0.81	±0.05
10–11	7	0.78	0.06	6	0.65	0.08
11–12	8	0.41	0.12	8	0.32	0.13
12–13	22	0.80	0.05	20	0.51	0.11
13–14	29	0.84	0.05	18	0.77	0.06
14–15	53	0.53	0.07	8	0.63	0.09
15–16	58	0.63	0.06			
16–17	14	0.34	0.13			
17–18	3	0.53	0.11			

CONCLUSIONS

1. The basal metabolic rate during later childhood (boys) shows an irregularity in its decrease, related to puberty.

2. The average heat production as expressed in Calories per square meter per hour for each age group is given in table 1 which may serve as a set of norms, with the possible exception in the upper and lower age groups.

We wish to thank Dr. W. G. Karr of the Department of Physiological Chemistry, University of Pennsylvania Medical School, for his valuable suggestions and criticisms.

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